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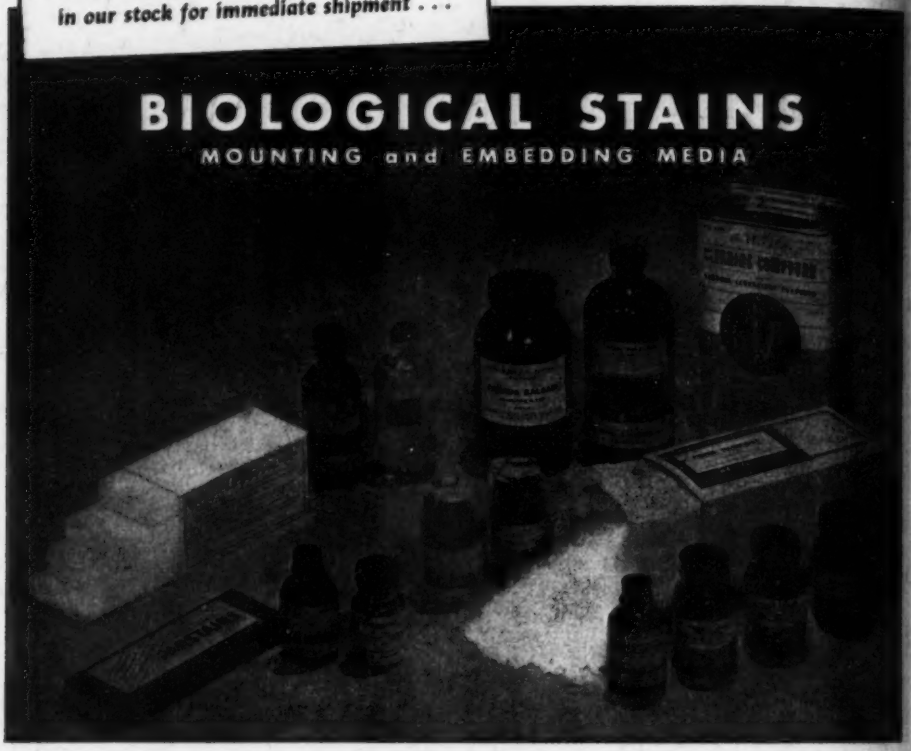

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THE
BIOLOGICAL BULLETIN

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THE INFLUENCE OF BODY WEIGHT, TEMPERATURE AND SEASON UPON THE RATE OF OXYGEN CONSUMPTION OF THE TERRESTRIAL AMPHIPOD, TALITRUS SYLVATICUS (HASWELL)

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Talitrus sylvaticus, a soil-inhabiting amphipod, (Family Talitridae), is widely distributed in the coastal region of eastern Australia and is particularly abundant in the litter layer of sub-tropical rain forests in this region. The experiments described here were designed to obtain part of the data necessary to estimate the quantity of energy which would have been used during a year by a population of *Talitrus sylvaticus* in a tract of rain forest at Bulli Pass, N.S.W. A preliminary study was made of the relationship between the rate of oxygen consumption of *T. sylvaticus* and body weight. This was followed by a study of the effect of temperature on the rate of oxygen uptake of *Talitrus* in both summer and winter.

Methods

Large numbers of *T. sylvaticus* were brought from the rain forest at Bulli Pass at regular intervals and kept in the laboratory in jars of leaf litter and soil at room temperature. Animals which had been in the laboratory longer than a month were not used in the experiments.

Rates of oxygen consumption were measured in a Warburg respirometer, the temperature of the water bath being controlled to within ± .01 of a degree. The volumes of the reaction vessels ranged from 3 to 4 ml.

The *Talitrus* used in any vessel were weighed *en masse,* then dropped into the vessel where they remained quietly on, or close to, two strips of moistened filter paper of known area which had been placed in the bottom of the vessel. In all experiments the vessels contained between 80 and 120 milligrams live weight of *Talitrus* and 0.8 ml. of 10 per cent KOH as an absorbent for CO_2. After each experimental "run" the animals were removed from the vessels and discarded.

Experimental Results

The effect of body weight at 25° C.

Figure 1 shows the relationship between the rate of oxygen uptake of *T. sylvaticus* and body weight at 25° C. The curve, fitted to the scatter of points by

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use of a double logarithmic regression, has the formula:

$$y = 533\, x^{-0.164},$$

where y is the rate of oxygen uptake in mm.^{3} O_2 per gm. per hour, and x is the body weight in milligrams. The rate of oxygen uptake, expressed as microliters of oxygen used per animal per hour by multiplying the right hand side of the above equation by x/1000, gave the formula:

$$y = 0.533\, x^{0.836}.$$

The effect of temperature

The relationship between the rate of oxygen uptake of *Talitrus* and temperature is illustrated by Figure 2. Figure 2 also shows the effect of the body weight

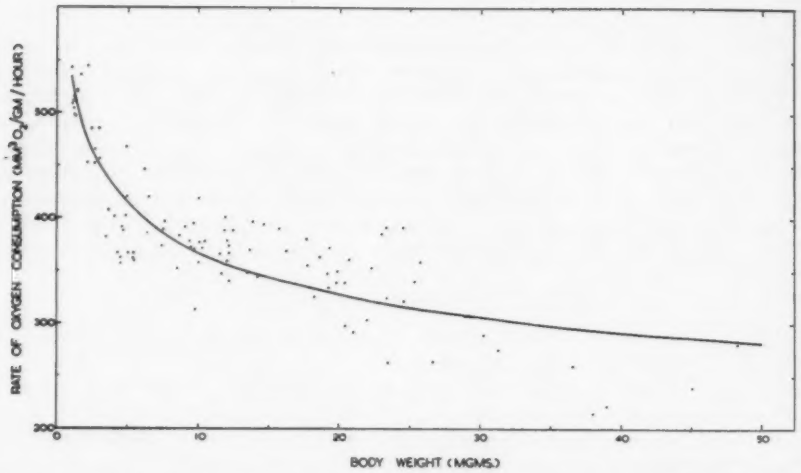

FIGURE 1. The relationship between the rate of oxygen consumption of *Talitrus sylvaticus* and body weight at 25° C.

of the animals (graphs A, B and C), and the effect of season on the rate of respiration of *Talitrus* over a range of temperatures.

A comparison of the graphs in rectangles A, B and C of Figure 2 shows that the rate of oxygen uptake of small *Talitrus* (mean body weight 1.5 mg.), was higher than that of medium and large animals (mean body weights 8.5 mg. and 21 mg., respectively), at temperatures above 15° C. The increase in the respiratory rate of the smallest animals, with increasing temperature, was more rapid than the corresponding increase in that of the largest animals. For example, between 20° C. and 30° C. the Q_{10} of *Talitrus* of mean body weight 1.5 mg. was 2.33 in winter while the Q_{10} of *Talitrus* of 21 mg. mean body weight was 1.66 in winter.

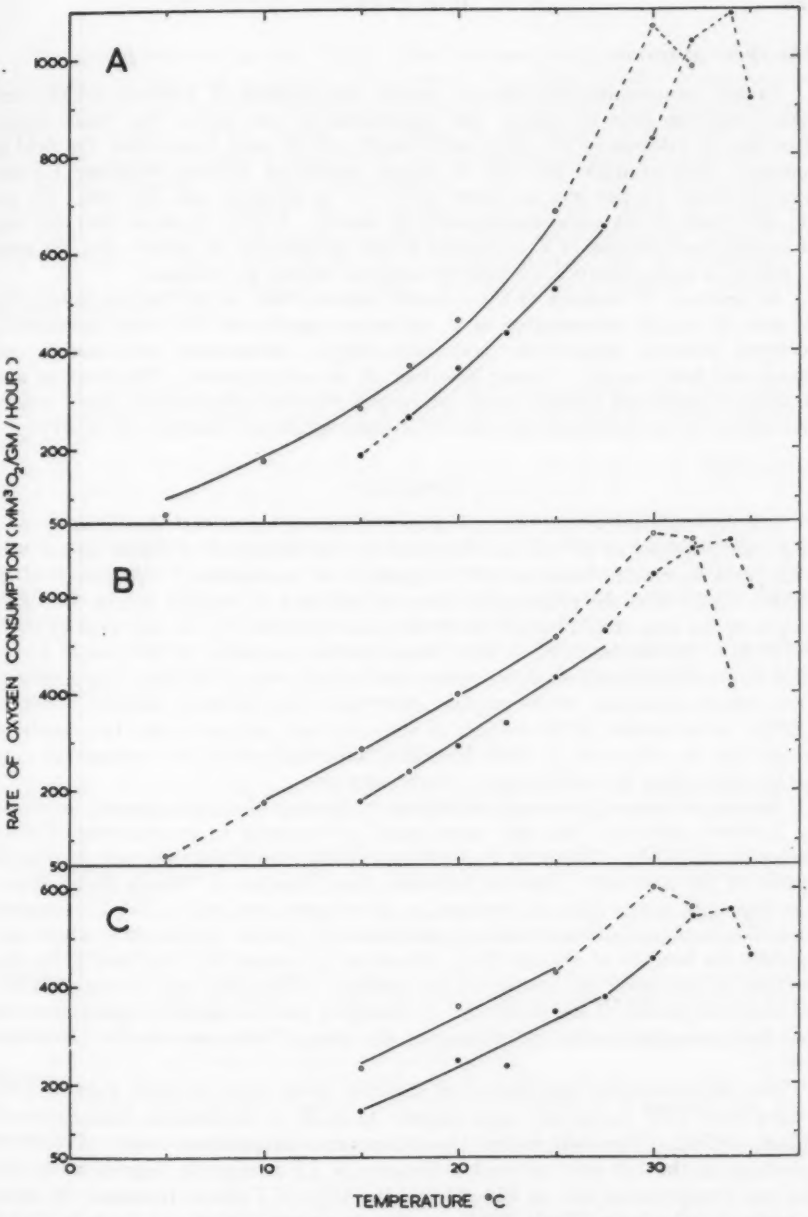

FIGURE 2. The relationship between the rate of oxygen consumption of *Talitrus sylvaticus* and temperature. Graphs A, B, C show curves for the rate of oxygen consumption of *Talitrus* weighing 1.5 mg., 8.5 mg. and 21 mg., respectively. Open circles, winter; black dots, summer. Broken lines are shown where the curves do not fit a logistic formula.

The effect of season

At any temperature the rate of oxygen consumption of *Talitrus,* which were taken from the field in winter, was approximately one and a half times higher than that of *Talitrus* of the same body weight which were taken from the field in summer. For example, the rate of oxygen uptake of *Talitrus* weighing 1.5 mg. was 187 $mm.^3$ O_2 per gm. per hour at 15° C. in summer, and 272 $mm.^3$ O_2 per gm. per hour at the same temperature in winter. Figure 2 shows that the rate of oxygen consumption of *T. sylvaticus* at any temperature in winter was the same as that at a temperature 2.5 centigrade degrees higher in summer.

An analysis of variance of these results showed that, of the factors influencing the rate of oxygen consumption of *T. sylvaticus,* significant first order interactions occurred between temperature and body weight, temperature and season, and season and body weight, P being less than .01 in each instance. The analysis also showed a significant second order interaction between temperature, body weight and season of the year upon the rate of oxygen uptake of *Talitrus,* ($P < .01$).

Discussion

The decrease in the rate of respiration of *Talitrus sylvaticus* relative to increase in its body weight at 25° C., as expressed by the exponent of 0.836, agrees well with previous results found on the respiration of crustaceans. Weymouth *et al.* (1944) found that the relationship between the rate of oxygen uptake and body weight of the kelp crab, *Pugettia producta,* was expressed by an exponent of 0.798 at 15° C. Scholander *et al.* (1953) found that an exponent of 0.85 could be applied to this relationship in many crustaceans ranging from the arctic fairy shrimp, *Branchinecta paludosa,* to the tropical giant land crab, *Sesarma ricordi.* Zeuthen (1953), in his review of the subject of body size and metabolic rate in organisms, stated that an exponent of 0.80 described this relationship for crustaceans containing more than 0.1 milligrams of body nitrogen.

The occurrence of a seasonal adaptation of the rate of oxygen uptake, as shown by *Talitrus sylvaticus,* has only been found infrequently in invertebrates (Scholander *et al.,* 1953). Edwards and Irving (1943a) found that the rate of oxygen uptake of the sand crab, *Emerita talpoidea,* from beaches at Woods Hole, Mass., was higher in winter than in summer at all temperatures below 20° C. In contrast, the amphipod, *Talorchestia megalophthalma* (family Talitridae), which also inhabits the beaches at Woods Hole, hibernates in winter and the rate of its respiration is the same in winter as in summer (Edwards and Irving, 1943b). Seasonal adaptation of metabolic rate to changing environmental temperatures has also been recorded in the gill tissues of the clam, *Venus mercenaria* (Hopkins, 1946).

The mean monthly temperature in the rain forest litter at Bulli Pass in 1951 ranged from 12.0° C. in July (mid-winter) to 21.0° C. in January (mid-summer) (Clark, 1954). The shift in the O_2 consumption-temperature curve of *Talitrus sylvaticus* to the left only covered a distance of 2.5 centigrade degrees along the abscissa (temperature axis in Fig. 2) and the R.Q. of *Talitrus* remained the same in summer and winter (Clark, 1954), so that its energy output was lower in winter than in summer.

The physiological mechanism which enables animals to alter their rate of oxygen consumption in response to season is unknown. Hopkins' (1946) demonstration of seasonal adaptation in the excised gill tissues of *Venus mercenaria* suggests that seasonal adaptation occurs within the tissues of the animal and hence may be effected by changes in the concentration of intracellular respiratory enzymes.

The author wishes to express his gratitude to Dr. L. C. Birch, Department of Zoology, University of Sydney, for his interest and advice during the course of this work, to the Research Grants Committee of the University of Sydney for their financial assistance, and to Mr. M. A. Bateman and Miss B. Cameron for their criticism of the manuscript. Thanks are also due to Miss W. Doran for drawing Figures 1 and 2.

Summary

1. The relationship between the rate of oxygen consumption and body weight of the terrestrial amphipod, *Talitrus sylvaticus*, was expressed by an exponent of 0.836 at 25° C.

2. The rate of oxygen consumption of *Talitrus sylvaticus* was significantly higher in winter than in summer at any one temperature.

3. Analysis of variance showed that, of the factors influencing the rate of oxygen consumption of *T. sylvaticus*, first order interactions occurred between temperature and body weight, temperature and season, and season and body weight. There was also a significant second order interaction between temperature, body weight and season on the rate of oxygen uptake of *T. sylvaticus*.

4. The adaptation of its rate of oxygen uptake by *Talitrus sylvaticus* to seasonal differences in temperature was not sufficient to enable it to maintain the same rate of output of energy in winter as in summer.

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PROTEASE IN AMMOCOETES ENDOSTYLE[1]

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It is now generally accepted that there is at least one proteinase associated with the breakdown of thyroglobulin in the thyroid gland. The action of such an enzyme is necessary to release thyroxine from protein, so that it may be able to diffuse through the follicular epithelium into the blood. The enzyme has been demonstrated in thyroids of rats and guinea pigs (Dziemian, 1943; De Robertis, 1941), man (De Robertis and Nowinski, 1946), and dog (Kamner *et al.*, 1950). From beef thyroid, Anson (1937) partially purified a proteolytic enzyme with properties of a catheptase; Weiss (1953) further characterized the beef protease biochemically. McQuillan and Trikojus (1953) were able to show the existence of a close thyroglobulin-enzyme association in hog thyroid and they devised a method for its purification.

Considerable interest attaches to the question of whether the larval thyroid gland of lampreys, the "endostyle," possesses a proteinase similar to that of thyroids of the higher vertebrates. The endostyle opens by means of a duct into the pharynx so that any thyroid-like secretion could be passed through it for digestion and absorption in more posterior parts of the gut. Thus, it would not appear to be essential for the endostyle to have an enzymatic thyroxine-freeing mechanism. On the other hand, when the lamprey larva undergoes metamorphosis, the duct to the pharynx is closed and the endostyle is in this way converted into a more or less typical follicular thyroid gland in the adult. Such closed follicles would require enzymatic hydrolysis of their contained thyroprotein. At what stage in these developments does the thyroidal proteinase appear? Does it precede the endostyle-thyroid transformation, or follow it? These are the questions that the present work has attempted to answer.

Materials and Methods

Because of the small size of the endostyle, it was necessary to employ a procedure especially adapted to the determination of the proteolytic enzyme on a micro level. The technic used for the measurement of enzyme activity was modified from the procedures of Anson (1937) and Kamner *et al.* (1950).[2] The animals used were the ammocoetes larvae of *Petromyzon marinus* collected in the Salmon River near Plattsburg, New York.

[1] We gratefully acknowledge the use of some facilities of the Brookhaven National Laboratory in the course of this work. This work was aided by Contract NR 163-208 between the Office of Naval Research, Department of the Navy, and Columbia University, and more recently by a grant from the National Science Foundation.

[2] We are grateful to Dr. L. C. Sze for his help in developing the microtechniques employed.

Larvae, ranging in size from 13 to 16 cm. in length, were used. The lower half of the pharynx was removed from unanesthetized animals and the endostyle was dissected out and weighed. Two endostyles at a time were homogenized in a glass microhomogenizer containing 50 microliters (μl) of 60% aqueous glycerol. Additional glycerol solution was added until the total tissue concentration was 0.1 mgm./μl. This homogenate was refrigerated for 15 minutes at 4° C., and then distilled water was added to give a final tissue concentration of 0.05 mgm./μl. After careful stirring with a fine, glass rod, the mixture was again refrigerated for one-half hour. The homogenate was then centrifuged, and the supernatant, used for analysis, was drawn off as needed. Endostyles of four or five ammocoetes were used in each separate test.

A 2.5% aqueous solution of hemoglobin,[3] was made up not more than three days before use as the stock substrate material. The final solution of buffered substrate, prepared just before use, contained 4 ml. of 2.5% hemoglobin solution and 1 ml. of a solution of acid (and/or salt) of desired molarity. The pH was determined on the Beckman pH meter.

TABLE I

The effect of hydrogen ion concentration upon endostylar proteolytic activity. Incubation time: 5 hours

25 μl of homogenate (1.25 mgm. tissue) +150 μl of buffered hemoglobin, pH:	Micrograms of tyrosine freed per 1.25 mgm. tissue
2.00	0
2.60	0
3.42	25.5
3.90	35.4
4.00	36.2
4.30	27.4
5.00	14.0

A series of conical two-ml. Pyrex centrifuge tubes, each containing 10 μl (or more) of the homogenate supernatant, was prepared in groups of three, two experimentals and one control tube. Into each was pipetted 150 μl of the buffered substrate of the desired pH. To the controls, 275 μl of 0.3 *M* trichloroacetic acid (TCA) also was added. This proved to be as satisfactory as heat inactivation suggested by Kamner *et al.* (1950). These tubes were then stoppered and placed in a water bath at 37.0° ± 0.02° C. After the desired interval, the reaction in the experimental tubes was stopped by the addition of 275 μl of 0.3 *M* TCA and thorough mixing. Since filtering of these small volumes was not practical, the tubes were centrifuged at 3000 rpm. and the clear supernatant was analyzed for liberated tyrosine as follows: 200 μl of the supernatant of each tube were quantitatively transferred into a correspondingly marked cuvette. To this, 400 μl of 0.5 N NaOH was added in similar fashion and the cuvettes were inverted three times to insure complete mixing. One part of Folin-Ciocalteu phenol reagent[4] was

[3] Powdered Bovine Hemoglobin Enzyme Substrate Powder, Pentex Incorp., Kankakee, Illinois.

[4] Eimer and Amend, New York.

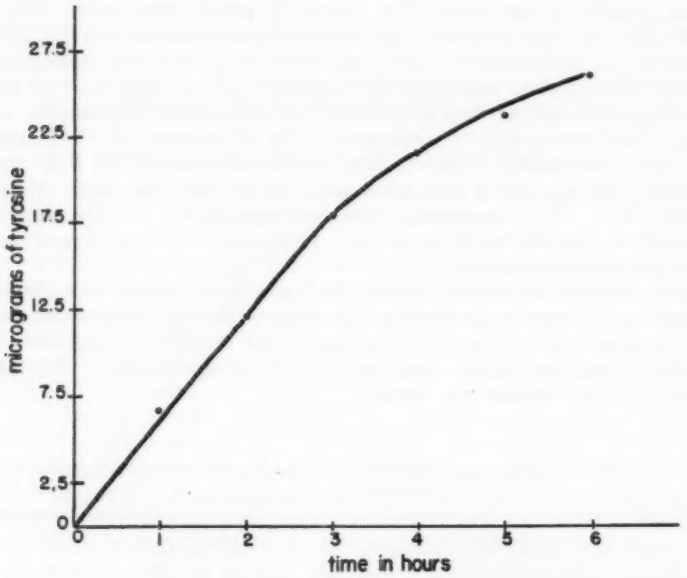

FIGURE 1. Proteolytic activity of endostylar enzyme as a function of time. Tissue concentration: 0.5 mgm./10 μl. of homogenate.

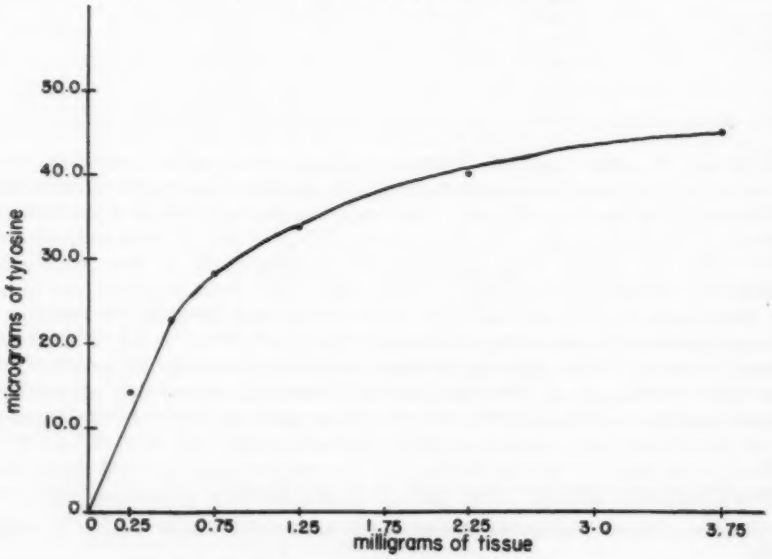

FIGURE 2. Proteolytic activity of the endostyle as a function of its concentration expressed as milligrams of tissue per unit volume. Incubation: 5 hours.

diluted with two parts of distilled water, and 150 μl of this dilution was added to each cuvette which was again inverted three times. The characteristic blue color produced in 15 minutes was measured in a Coleman, Jr. spectrophotometer at 700 λ and values were expressed as micrograms of tyrosine liberated by the homogenate after subtraction of the control values. The tyrosine standardizing solution was prepared from l-tyrosine, and the curve was made by reading spectrophotometric values corresponding to quantities ranging from 5 to 35 micrograms of tyrosine.

Results

The proteolytic activity of glycerol extracts of endostylar tissue first was measured as a function of the hydrogen ion concentration of the *in vitro* reacting system. Optimal activity for digestion of the hemoglobin substrate was found at pH 3.9–4.0, decreasing measurably on either side of this peak (Table I). It is interesting that this optimum is quite close to those found by others in studies of mammalian thyroidal protease.

To make possible further comparison of the ammocoetes endostylar protease with the thyroidal proteases of other animals, studies were made of the proteolytic activity of the enzyme as a function of time, and of its concentration. The data for both of these studies were confirmed by several repetitions, and two series of experiments are summarized in Figures 1 and 2. It may be seen that under the conditions employed, and within stated limits of time and enzyme concentration, proteolytic activity is a linear function of either of these two variables.

Discussion

Schneider (1879) first observed the histological changes during the transformation of the endostyle of metamorphosing larvae, and these observations led him to the conclusion that the endostyle is the forerunner of the well-defined thyroid gland of the adult. It was Dohrn (1875, 1886) and later, Leach (1939), who suggested that the endostyle might serve functionally in the production of a hormone or its precursor. They further believed that this secretion might pass through the pharynx to the alimentary canal where it could be absorbed. Recently, Sterba (1953) in an extensive study determined that the Type III secretory cells of the endostyle of *Petromyzon planeri* contributed to a large extent to the formation of the follicles of the adult thyroid. This supplied the basis for the correlation of morphology and function, for it was the Type III cells of *Entosphenus lamottenii* that accumulated radioiodine in radioautographic tests (Gorbman and Creaser, 1942). This ability of the endostyle to metabolize and store iodine demonstrates its thyroid-like function, even during the prolonged larval period. Further confirmation has been provided by Leloup and Berg (1954) who showed by means of radiochromatography that the endostyles of the larvae of *Petromyzon marinus* accumulate iodide, and form moniodotyrosine, and diiodotyrosine when maintained at 15° C. and kept at 2° C., they also form thyroxine. Evidently, the same cells that participate in follicular formation of the adult gland are able, long before, to elaborate the precursors of thyroxine as well as thyroxine itself.

Following Dohrn's suggestion (1886) that the endostyle is an organ of digestive secretion, Alcock, as early as 1899, tested the endostyle for ability to digest

fibrin. Although she found other parts of the digestive tract active in this respect, the endostyle did not, in her tests, digest fibrin. She concluded that the endostyle did not function as a source of digestive ferments. Since this negative report there has been no work produced to either confirm or contradict this conclusion. The data reported here seem to establish the presence of an active protease of the catheptase type in the endostyle of ammocoetes. Further, this enzyme is present in a concentration comparable to the protease in the thyroids of mice and adult lampreys (unpublished data).

The usefulness of the endostylar protease is difficult to assess in terms of thyroid function. Whether the endostylar secretion, particularly that of the iodine-metabolizing Type III cells, passes into the lumen by holocrine (Gorbman and Creaser, 1942) or merocrine (Sterba, 1953) means, it seems to move rapidly into the pharynx (Sterba, 1953). This would not seem to provide sufficient opportunity for enzymatic hydrolysis of the secreted iodoprotein. If not digested in the endostyle it appears more likely that digestion of the iodoproteins would be continued and completed in the intestine, and it would be followed by absorption of the hormone.

At any rate, it is reasonable to conclude that the thyroidal protease of lampreys is formed in the larval endostyle, and therefore it precedes follicular differentiation, when its presence would be obligatory for proper thyroidal function.

Summary

1. A proteolytic enzyme was demonstrated, by use of a new microanalytical technique, to be present in the endostyle of the ammocoetes larva of *Petromyzon marinus*.

2. The protease has an optimum pH of 4.0. The hydrolysis of hemoglobin (expressed as micrograms of tyrosine released) under the conditions employed, and within a period of three hours, is a straight line function of time. The hydrolytic activity of the endostylar enzyme is also a straight line function of enzymatic concentration, but within restricted and defined limits.

3. The probable site of action of the hydrolysis of the endostylar thyroprotein is discussed in regard to the usefulness of the investigated endostylar protease.

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INITIAL FLIGHT DIRECTIONS OF HOMING BIRDS

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Our understanding of the orientation of birds has been significantly increased by recent experiments in which Kramer (1952, 1953) and Matthews (1953a, 1953b) have demonstrated that homing pigeons, and probably Manx shearwaters as well, can choose approximately the correct homeward direction during the first few minutes after release in unfamiliar territory. Yet such a high order of homing ability is not invariably displayed, nor is a nearly correct heading always followed by a rapid return to the nest. For when certain birds are released in unfamiliar territory they tend to fly in a particular direction even though this is not the homeward direction (with pigeons it may be the direction of previous training flights). Still other species, such as gannets, do not seem able to choose any consistent direction at all (Griffin and Hock, 1949). For convenience these three kinds of orientation have been called types III, II, and I, respectively: type I being reliance on landmarks with wandering or exploration in unfamiliar territory, type II the tendency to fly in a particular direction which may differ from the actual homeward direction, and type III the ability to fly in roughly the homeward direction over unfamiliar territory, regardless of what that direction may be (Griffin, 1952). Type II and III homings seem to occur only under clear skies, so that the sun is probably one important factor enabling the birds to choose a particular direction.

In seeking a general understanding of the orientation of birds it is of obvious interest to inquire which species show each of these three types of homing, and under what conditions. Chiefly in the hope of finding a species which would consistently display type III homing, the initial flight directions of common terns (*Sterna hirundo*) and Leach's petrels (*Oceanodroma leucorhoa*) were studied by methods designed to obtain accurate information about each bird's direction of flight away from the release point as long as it could be seen through binoculars. The results are of interest, even though type III homing was not demonstrated, because the common terns showed a consistent tendency to fly in a southeasterly direction when released inland, even when home lay in a different direction.

We wish to express our indebtedness to many friends who assisted in catching or transporting the birds, in watching for returns at the nests, or during the actual observation of initial flight directions. We are also grateful to the Office of Naval Research which provided financial support through research contracts with Cornell and Harvard Universities, and to the Marine Biological Laboratory at Woods Hole, Mass. which provided facilities and boat transportation during 1952 and 1953.

Methods

Incubating birds were captured at their nests and set free at inland release points chosen to facilitate observation of the initial flight direction. Since earlier homing experiments had shown that the recorded homing times were far too long to establish direct homeward flights (Griffin, 1940, 1943), in only a few of the present experiments was an observer stationed at the nests to record the time of each bird's return. The methods of capture and observation at the nest were the same as in previous homing experiments with these two species. All birds were transported in boxes through which they could see nothing of their surroundings, and experience showed that the use of large boxes holding several birds enabled them to reach the release point in better condition than smaller individual compartments where the feathers were disarranged by rubbing against the walls. Water was provided every few hours, especially in hot weather, and most of the terns seemed to take advantage of it both for drinking and bathing. The birds were released with a minimum of handling, usually being allowed to take wing spontaneously. Each bird was released alone and not until some minutes after the previous one had been lost to view through the binoculars. Except for occasional instances noted below, each bird's flight thus represented an independent choice of direction.

During 1952 and 1953 most of the birds were observed through hand-held binoculars which allowed us to determine only the final bearing of the bird from the release point, the direction which Matthews calls the "vanishing point." Since in many cases the bird's bearing shifted steadily during the last minute or two it was clear that its actual direction of flight was different from the final bearing from the release point. In 1954 binoculars were therefore mounted on tripods equipped with alidades so that we could note the actual bearing throughout the observations. In all but the first experiment in 1954 two such alidades were stationed ¼ to ½ mile apart, one with 7 × 50, the other with 15 × 60 mm. binoculars. An assistant noted the bearings every 15 seconds, giving warning to the observer before each reading so that he could center the bird in the field of the binoculars. This procedure gave simultaneous cross bearings accurate to about one degree, so that the bird's actual flight path could be plotted for several minutes.

The flight paths plotted in Figure 2 are typical of the results obtained under clear skies, and in many cases the actual flight directions plotted in this fashion differed significantly from the bird's final bearing as seen from the release point. Even after one observer had lost sight of a bird the successive bearings from the other alidade allowed us to extrapolate the probable flight path as the bird receded into the distance. This was done by assuming that the actual distance flown during each 15 seconds remained constant; or, if the bird could be seen to be circling, it was assumed to cover less distance. Such extrapolated flight paths based on a single set of bearings are shown in Figures 2 and 3 by open circles or triangles connected by broken lines, while solid circles and triangles connected by continuous lines represent positions determined by cross bearings.

The selection of release points was of crucial importance. Not only was it essential to have a clear view in all directions, but it proved advisable to avoid the proximity of lakes or streams. Especially in cloudy weather the terns tended to alight on small ponds or streams within a mile or so of the release point. None

of the release points was closer than 15 miles to the ocean, nor closer than ten miles to large lakes or rivers. These requirements severely limited the choice of release points in New England, but two nearly ideal sites were found at Storrs, Connecticut, and Houlton, Maine.

Initial Headings of Common Terns

The results of fifteen releases involving a total of 145 terns are summarized in Table I, while Figure 1 shows the locations of the release points and nesting colonies (Penikese Island, Mass., and North Sugarloaf Island, Maine). Wind direction is shown in Table I as downwind, *i.e.*, the direction to be expected if a bird merely

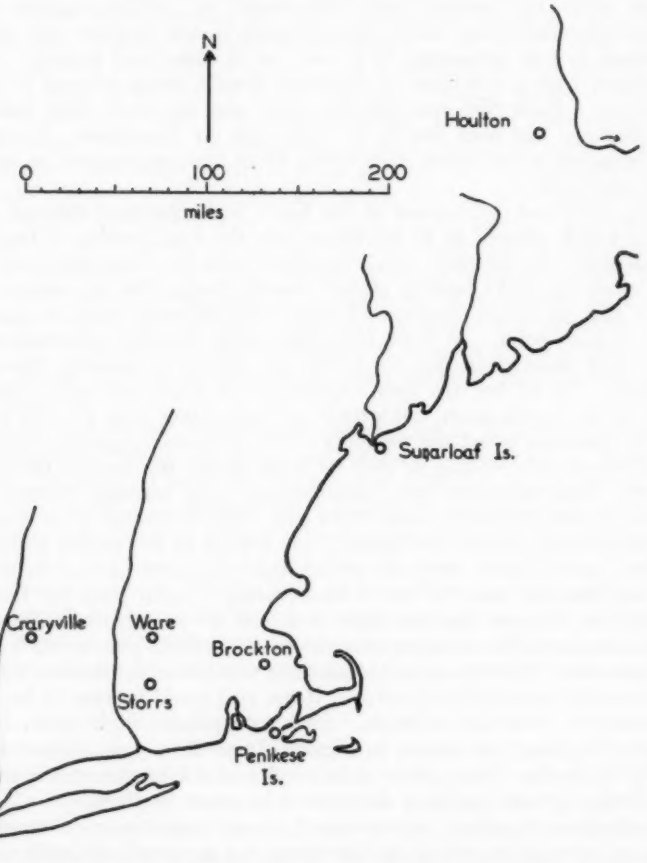

Figure 1. Map showing nesting colonies and release points (small circles). Birds from Sugarloaf Island are called "Me. birds" in Figures 2 and 4; birds from Penikese Island "Mass. birds."

TABLE I

Initial flight directions of common terns released inland. All birds from Penikese Island, Mass., except those noted as "Me. terns" which had been caught on Sugarloaf Is. (see Fig. 1). Brackets in right hand column indicate birds under shifted light schedules (see text)

Date	Release pt.	Down wind direction, velocity	Sky cond. and sun visibility	Home		Observed flight headings	
				Dist. (miles)	Bearing (degr.)	Mean	Individual terns
29–5 1954	Storrs, Conn.	N–NNE, 10–20	Br. clds., sun vis.	75	115	140	131, 185, 123, 105, 126, 142, 131, 135, 180
8–7 1954	Storrs	E, light	Br. clds., sun vis.	75	115	142	161, 146, 138, 140, 130, 154, 143, 127
8–7 1954	Storrs (Me. terns)	E, light	Br. clds., sun vis.	179	44	142	161, 151, 135, 127, 137, 144, 165, 90, 145, 180, 124
19–7 1954	Houlton, Me. (Me. terns)	N–NW, light	Overcast, some rain	190	211	—	6, 278, 42, 71, c110, 172, 105, c45, 171
28–7 1954	Houlton (Me. terns)	NE–SE, light	Clear	190	211	149	147, 187, 153, 133, 175, 133, 167, 144, 150, 129, 127, 154, 120, 160, 154
29–7 1952	Brockton, Mass.	W to NW, light	Sun visible	41	172	200	SSW, SSE, SSW, NNW, SW, SSE, SSE, SSW, SSE
6–8 1952	Brockton	S, light	Overcast, light fog sun not vis.	41	172	—	(E), (SE), (ENE), (S), (SW), (NE), (NE), (S), E, ENE, NE, W, SSW
6–7 1953	Brockton	E, 5–12 m.p.h.	Clear	41	172	151	E, SSW, S, S, S, ESE, SSE, ESE, SSE, SE, SSW
20–7 1953	Brockton	NW, light	Hazy, sun visible	41	172	158	SSW, E, S
22–7 1953	Brockton	S–SSW, 5–8 m.p.h.	Fog, sun not vis.	41	172	210	S, SW, SW
9–7 1953	Ware, Mass.	SSE light to mod.	Clear	87	131	116	E ESE, (E), (SE), E, (SE), (SSE), (S), (E), E, SE, ESE, E
23–7 1953	Craryville, N. Y.	Calm	High solid overcast	146	111	87	SSE, SE, NE, SE, (E), (NE, NE, NE) (see text)
9–7 1954	Craryville	N, very light	Clear	146	111	159	136, 160, 214, 145, 146, 155, 149, 170
9–7 1954	Craryville (Me. terns)	N, very light	Clear	222	60	131	120, 168, 119, 170, 151, 155
21–7 1954	Craryville	S–E, light	Br. clds., sun vis.	146	111	175 (most flew to lake)	185, 182, 175, 217, 189, 163, 181, 179, 178, 177, 161, 164, 184, 170, 149, 176, 173, 159, 154

drifted with the wind. Our best estimate of the general trend of each bird's flight after it had begun to head across country is listed in the right hand column of the table, in compass directions when hand-held binoculars were used, in degrees when alidades were employed. The actual precision of these estimates varied

widely, depending on the straightness of the bird's flight path, but when two alidades were used, almost all the estimates were accurate within ± 5°. Every bird for which any sort of meaningful initial homing direction was obtained is included either in Table I or in the section entitled "Miscellaneous Release Points." The only birds omitted from Table I were 20 lost to view (behind trees, against the sun, etc.) while still close to the observer, and 26 birds seen to land on ponds or other local bodies of water. Even these birds showed a southeasterly trend, so that their omission has not removed data inconsistent with the conclusions reached below.

The general behavior of the terns immediately after release was to circle or fly in irregular patterns with frequent changes of direction for about one to three minutes. Then, in good weather, they would generally begin flying an essentially straight course which soon carried them out of sight, although circling might continue at intervals. Examples of such reasonably straight flights are shown in Figures 2 and 3, with the initial circling omitted. When the individual points (one plotted for every 15 seconds) are close together the bird was circling. The time during which each bird was observed varied from four to twelve minutes, and most of them were lost to view at one to two miles from the release point. On some days the terns climbed on updrafts and even soared briefly, so that altitudes of a few hundred feet were reached. Our vertical bearings were only accurate enough, however, to convince us that neither at Storrs, nor at the other release points except possibly Brockton and Chester, could any of the birds have seen the coast while under observation.

Storrs, Connecticut

Consistent and significant headings were observed in all experiments at this release point. In a preliminary test of five terns from Penikese Island, two could be seen to head south or southeast, the others being lost to view before they showed any definite headings. Penikese Island is 75 miles from Storrs at 115°.

On May 29, 1954 a group of 16 terns was brought from Penikese Island to Storrs in excellent condition and watched for periods ranging up to 12 minutes with a single pair of binoculars mounted on an alidade. Although the first six birds released were lost behind trees before they had shown definite headings, we were then able to shift the observers' positions so that nine of the remaining ten could be followed for considerable distances. Despite a brisk wind from the south these birds showed a striking tendency to head southeast, their final bearings varying from 117° to 180°, and when reasonable corrections were made in the cases of four birds for which the bearings were shifting significantly during the last minute or two of observation, the estimated headings became those listed in Table I, with an average of 140°.

To determine whether this was a true case of type III homing, two groups of terns were next brought to Storrs from the Maine and Massachusetts colonies shown in Figure 1. On July 8, 1954 these 23 birds were released singly and in an irregular sequence not known to the observers by Dr. J. Manter of the University of Connecticut. Two observers stationed 2500 feet apart watched through binoculars mounted on alidades, and all but two terns showed definite headings while observed for periods ranging from 4 to 7½ minutes. All of these bearings

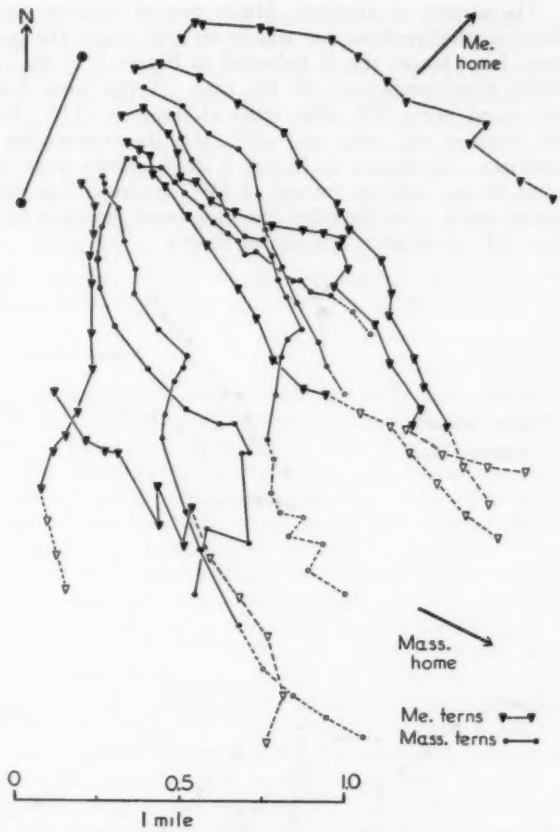

FIGURE 2. Flight paths observed after releases at Storrs, Conn., July 8, 1954. Solid triangles and circles stand for cross bearings, open triangles and circles for single bearings. The baseline connecting the observer's positions is shown in the upper left hand corner. Note that birds from the Maine and Massachusetts colonies show no significant difference in headings.

are included in Table I, and eleven of the longest series of observations are plotted in Figure 2. The flight paths omitted from Figure 2 were those which covered shorter distances, involved a small proportion of double bearings, or simply fell too close to one or more of the lines already drawn in this figure. These results demonstrate clearly that there was no difference in headings between the Maine and Massachusetts birds, both averaging 142° although the home directions were 44° and 115°, respectively.

Houlton, Maine

To analyze this southeasterly tendency further we next selected a release point at which the homeward direction was as different as practicable from those al-

ready tested. The airport at Houlton, Maine proved suitable since it provided excellent visibility in all directions and was in an area where the general drainage pattern was away from home; this is indicated in Figure 1 by the St. John River to the east which flows southward off the map. Terns from Sugarloaf Island were used, this island being 190 miles from Houlton at 211°. For the release on July 28 the weather was clear and mild, and the observation times ranged from 4 to 9 minutes. As shown in Figure 3 the headings were very similar to those observed at Storrs, with an average of 149° in comparison with 142° at the Connecticut release point. On the other hand the home direction for the Sugarloaf Island birds was 211° at Houlton and 44° at Storrs.

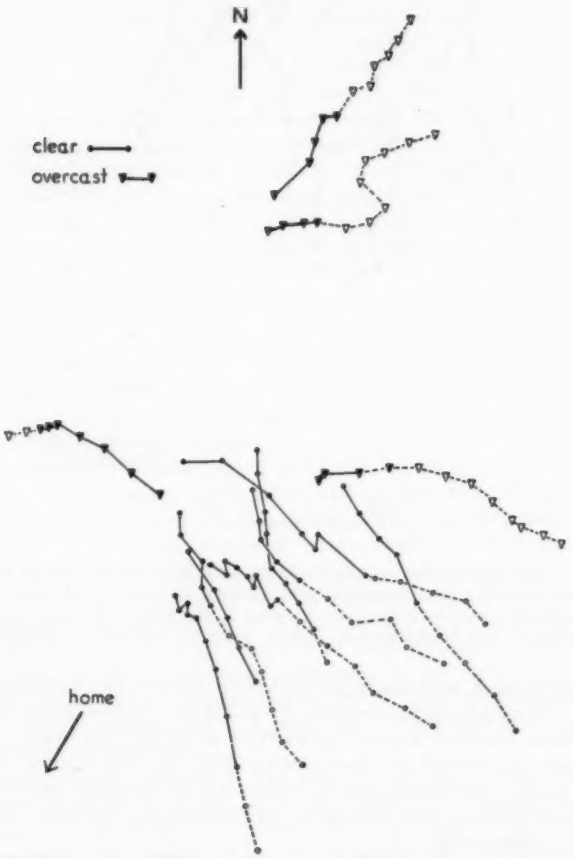

FIGURE 3. Flight paths observed after releases at Houlton, Maine on July 19, 1954 (heavy overcast and rain), and on July 28 (clear). Observers' positions not shown because they differed on the two days, being 3300 feet apart on July 19 and 2000 feet apart July 28. Scale and designation of cross and single bearings same as for Figure 2.

When terns from the same colony were released at Houlton under a heavy overcast on July 19 they obviously did not show the same tendency to fly southeast. Just as other terns had done at Craryville, Brockton, and elsewhere on overcast days, they showed much more hesitation and continued the circling phase of their flight for much longer than when the sun was visible. Observation times ranged from 4 to 10 minutes, but the birds did not fly as far from the release point as on the 28th. Eight landed on a small pond to the north of the airport, and these are not included in Table I. Indeed only 4 out of 20 showed a consistent enough heading to justify plotting their flight paths in Figure 3. The birds included in Table I and Figure 4, but not in Figure 3, were either followed by only one observer or yielded too few bearings to indicate a definite trend; their final bearings from the release point are the values listed in Table I.

Miscellaneous release points

Several other releases led to results consistent with those described above, and they are included in Table I or listed below even though they involved less satisfactory conditions. Those birds indicated in Table I by bearings in brackets were subjected to shifted schedules of light and darkness during two to four days in captivity in an attempt to shift their activity rhythms. No effect of this treatment could be discerned in their headings, but it is doubtful whether the treatment was long enough in duration to actually alter the activity rhythms.

Several groups of terns were released at the small airport south of Brockton, Mass., although the view is restricted by buildings and trees and Brockton is too close to the ocean to be certainly in unfamiliar territory. The time of day varied widely in the various releases, as did the observation time, but neither could be correlated with the initial headings. The results at Brockton were merely suggestive of a southerly (and approximately homeward) heading under clear skies, with a much wider scatter under a heavy overcast. A hilltop near Ware, Mass., was used only once because trees restricted the view and because a large pond was visible to the east, towards which several of the birds headed.

Eight terns were released at Craryville, N. Y., on the afternoon of July 23, 1953 under heavily overcast skies with light intermittent rain. The three birds indicated as "(NE, NE, NE)" had first flown off to the west, two of them remaining out of sight for several minutes. Several minutes later these two returned to the vicinity of the release point, were joined by the third, and then all flew off northeast together. While driving west towards the Hudson River shortly after releasing these birds it became obvious that the overcast extended only about five miles to the west of the release point and that the first two terns might have seen the afternoon sun during their flight to the west. Two releases at Craryville in 1954 confirmed the observations at Storrs, as is shown in Table I. The observers' alidades were 1300 feet apart, and the observation times ranged from 3½ to 9 minutes on July 9 and from 3½ to 7½ minutes on July 21st. But the results are complicated by the presence of Copake Lake 1½ miles south of the release point subtending an angle of 34° (156°–190°). On July 9 only about half of the birds seemed to be heading towards this lake, but on the 21st almost all did so, and we are certain that several actually landed on it.

In 1953 three terns were released at Keene, N. H., and two south of Barn-

stable, Mass., in both cases under heavy clouds with rain. Two of the Keene birds and one of the Barnstable terns landed on a pond; the other Keene bird flew west; the second Barnstable bird south. A group of seven terns was released July 9, 1953 in clear weather at Brookfield, Mass.; one flew to the east, while the others landed on a creek near the release point. Four terns from a colony at Plymouth, Mass. were released in clear weather at Chester, N. H., their nests being 75 miles away at 155°. Two were lost to view behind trees before choosing a definite heading, but the others were observed with two alidades for about six minutes and headed at approximately 214° and 145°, respectively. Unfortunately, the topography of this area, together with the altitude reached by these birds during our observations, might have permitted them to see the coast.

Initial Headings of Leach's Petrels

One petrel taken from Penikese Island to Storrs was observed to fly for some distance in a generally correct homeward direction. Twelve others from Kent Island, near Grand Manan, New Brunswick were released at Brockton and they also headed towards home. But these birds were rather weak and the homeward direction was downwind. The sun was visible during both these observations. Encouraged by these preliminary results, on June 19, 1954 we carried 22 petrels from Little Duck Island, near McKinley, Maine, to Houlton, Maine. They were released in excellent condition, some having been in captivity only eight to ten hours and the rest about 24 hours. There was a light wind blowing from the south. Ten petrels were lost to view soon after release, but satisfactory observations of a reasonably straight flight away from the release point were obtained for the other twelve. During six of these observations the sun was visible, for the other six it was hidden by clouds. Our best estimates of the final flight directions of the birds which could see the sun were: 27°, 105°, 205°, 226°, 330°, and 340°, and the six which could not see the sun showed headings of 15°, 15°, 28°, 31°, 280°, and 316°. Since the home island was 141 miles away at 189° there was no apparent tendency to fly towards home or in any other single direction. The northerly tendency of the birds released under overcast skies was possibly due to the presence of a small pond in that direction. This lack of any consistent directional trend after the release at Houlton, together with the greater difficulty of observing petrels at a distance, discouraged us from further attempts to study their initial headings.

Homing Performance

Earlier homing experiments with terns and petrels had shown that homing was relatively slow; hence in only a few of the present experiments was an observer stationed at the nesting colony to record the time of each bird's return. The following results of these experiments confirmed the previous conclusion that the homing times were far too long to establish whether or not direct homeward flight had occurred. After the release of terns at Brockton from 9:20 a.m. to 12:30 p.m. on July 6, 1953 a careful watch at ten of the nests showed eight returns by the end of the day following release. The most rapid was a tern that showed an initial heading of SSE (home being 41 miles away at 172°), and reached its nest in 3 hours 21 minutes. This corresponds to a direct flight at

12.3 m.p.h. The other birds were not nearly so rapid, however, the return times and initial headings being: 7 hours (SE), 20 hrs. (S), 24 hrs. (S), 27½ hrs. (SSE), 30 hrs. (S), 30 hrs. (ESE), and 31 hrs. (SSW). A less thorough watch for returns was maintained at Penikese Island after the July 9 release at Ware, and three returns arrived 16, 18, and 18 hours after release. The earlier homing experiments with terns had included one group released inland at 95 miles northwest, and return times ranged from 10 to 80 hours.

Of the 22 petrels released June 19 and 20 at Houlton, five reached their nests 141 miles away during the period from June 21 to 24 when the burrows were checked daily. Three of these birds had been released at Houlton late on the afternoon of June 19; one returned during the night of June 21–22, the other two the following night. One of the latter was the only return for which a satisfactory observation of initial flight direction had been obtained—330° in contrast to the true homeward direction of 189°. In previous homing experiments with this species a similar proportion of returns was recorded within four or five days after releases 120–170 miles at sea or along the coast.

Discussion

It seems clear that when these terns were released inland under clear skies (and were not attracted to local bodies of water) they tended strongly to fly in a southeasterly direction. This orientation was not appreciably affected by a wide variety of wind directions, and it was independent of the time of day. For instance in the four most significant experiments the actual times of release (EST) were as follows: Storrs, May 29, 2:00–5:15 p.m.; Storrs, July 8, 10:48 a.m.–3:56 p.m.; Craryville, July 9, 7:46–10:28 a.m.; and Houlton, July 28, 7:23–11:53 a.m. Yet there was no significant difference in the headings on these four dates, nor between the earlier and later releases on a given day. The release points at Storrs, Houlton, and Craryville were almost certainly in unfamiliar territory. This is true in the sense that recognition of landmarks during the first few minutes after release seems highly improbable, even though many hours of exploration might eventually have brought the birds to familiar territory. Since the headings of the Maine terns were clearly different from the homeward direction, we are evidently dealing with an example of type II orientation—a tendency to head in a certain direction whether or not this carries the birds towards home.

These experiments also indicate that the southeasterly orientation requires a view of the sun. But our data supporting this conclusion are less extensive than those demonstrating the consistency of the headings when the sun is visible. In one exceptional case (Brockton, July 22, 1953) three terns were released in a fog when the sun was not discernible to a human observer, and they headed in the same general direction that other terns had chosen at this release point in clear weather. The most satisfactory experiments, with respect to numbers of birds and good conditions for observation, are summarized in Figure 4, where the nearly random scatter under a heavy overcast is compared with the southeasterly tendency in clear weather. It is of some interest to note that while Matthews reports that as little as 50% cloud cover caused marked deterioration in the homeward orientation of Manx shearwaters, these terns continued to show equally consistent headings even when the sun could be seen only occasionally between breaks in a 90–

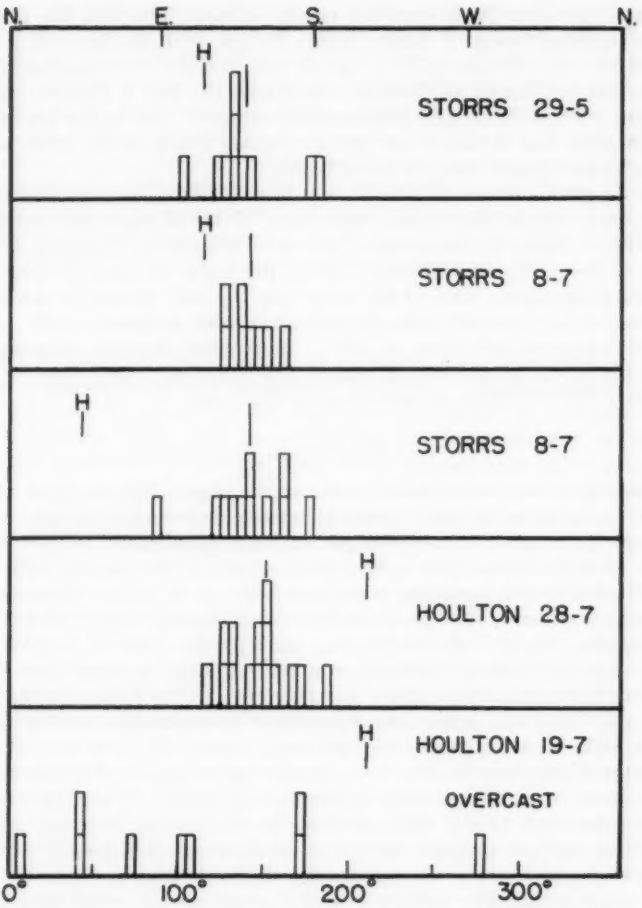

FIGURE 4. Graphic comparison of initial headings of 53 terns during the 1954 experiments at Storrs and Houlton. Homeward direction indicated by "H," mean heading of each group by vertical line. Sun visible to all birds except the last group labelled "overcast." Note the nearly identical average headings despite the 167° range of homeward directions.

95% cover of broken cumulus clouds. Only under a complete and thick overcast such as that prevailing at Houlton on July 19, 1954 did we observe a significant departure from the southeasterly headings.

Type II homing has been noted both in pigeons (Hitchcock, 1952; Matthews, 1951) and in certain wild birds (Rüppell, 1944; Rowan, 1946). Its appropriateness can be understood when the direction of flight corresponds to the previous training flights of pigeons or to the migration route which a given species can be ex-

pected to follow at the time of the experiment. But why do common terns tend so strongly to fly southeast? The migration route of this population of terns has been analyzed by Austin (1953) on the basis of a large number of recoveries of banded birds. This evidence could be interpreted as showing either that these terns first fly southwest along the coast and then move southeast to the general area of Jamaica, Trinidad, and Venezuela, or that they fly roughly south southeast across the ocean from their breeding grounds to their winter range. The initial headings we observed are thus similar to the direction of the fall migration; but our experiments took place during the nesting season when it is difficult to see why a tern should begin to migrate. It seems more likely that the observed headings represent a tendency to fly towards the coast. These terns do fly inland from time to time, and an ability to head towards the southeast would serve to guide them quickly back to the coast after any excursion inland from their normal summer range. This hypothetical explanation of the southeasterly headings also leads to the prediction that terns nesting in a different geographical area, such as the Great Lakes for example, would not show the same initial headings when released in unknown inland surroundings. We should thus like to suggest in closing that others interested in the phenomena of bird orientation attempt to repeat this type of observation with the same or similar species nesting in other regions, and with other species that have not yet been observed carefully as they begin homing flights over unfamiliar territory.

Summary

1. The initial flight directions of common terns and Leach's petrels were observed for as long as possible after release in unknown, inland areas. In some experiments two observers obtained cross bearings on each tern for as long as it could be seen through binoculars (maximum 12 minutes and two miles distance).

This procedure is strongly recommended whenever the initial headings of birds are of interest, since it reveals the actual direction of flight with greater accuracy than recording only the "vanishing point."

2. The petrels exhibited no consistent headings towards home or in any other direction, but one experiment resulted in returns from an inland release point similar in speed to those reported earlier from releases at sea or along the coast.

3. The terns showed a consistent tendency to head approximately southeast when the sun was visible. Average headings in the most satisfactory experiments were 140°, 142°, 142°, and 149°, while the total range of headings among 43 terns in these four experiments with the sun visible was 97°. The mean deviation from the average heading of 144° was only 16°. There was no significant difference between the headings when the direction of home was 44°, 115°, or 211°.

4. This southeasterly tendency disappeared almost entirely when the sun was hidden behind thick clouds, but it persisted under 90–95% cloud cover when the sun could be seen occasionally through breaks in the clouds.

5. The southeasterly tendency represents a special type of what has previously been called type II orientation, and it is probably based upon the sun. It may be a useful ability for terns that nest along the eastern coast of the United States, since whenever they find themselves inland, flight to the southeast will bring them quickly back to the coast.

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X-RAY EFFECTS ON ADULT ARTEMIA

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The available information concerning the lethal dosage of ionizing radiations in animals is limited to a certain few microorganisms, selected insects and laboratory types of vertebrates. Even more limited has been research on radiation-induced sterility in females. Because there are extreme differences between vertebrate results and those with holometabolous insects, it is of interest to fill in the gaps by extending research to a wide variety of representative animals. The present paper introduces a member of a primitive order of crustacea to this form of research. Except for attempts to induce visible mutations (Gajewskaja, 1923), we have been unable to find previous reports of a radiobiological nature on Artemia.

Materials and Methods

Commercial, dry eggs of the California strain (diploid amphigonic) of *Artemia salina* Leach, obtained from the M.B.L. Supply Department, were hatched in sea water and reared to maturity in large battery jars. Evaporation in our basement laboratory at Woods Hole was extremely slight; replacement was with sea water as is the standard procedure in bulk culturing. Cultures were fed daily with a few drops of either homogenized liver paste (commercial baby food) or yeast suspension unless the growth of natural microorganisms was obviously abundant. As soon as sex characteristics were distinguishable, individuals were segregated into male and female holding jars. When the accumulated total was adequate to provide five or more groups of at least five adult animals, experiments were set up. In this way animals were used within three days after reaching maturity.

The x-ray generator was that of the M.B.L., Woods Hole. This has two water-cooled G. E. (XTP) tubes in opposed position and operates at 30 ma and a 200 kv peak with an inherent filter equivalent to 0.2 mm. of copper. Air circulation in the room is assured by a fan.

Artemia were exposed 5 at a time in small plastic containers (4000 cu mm.). Exposures below 5000 r were made at an intensity of 2550 r per minute. Higher doses were delivered at 6120 r per minute. Calibration was achieved by M. Berman by inserting a rubber-covered ionization chamber through a hole bored in the side of one of the plastic containers which was then filled with sea water. Control samples of Artemia were held in exposure chambers in the anteroom for a period equalling that of the longest experimental treatment.

After irradiation, Artemia were maintained in a 30° C. incubator where evapo-

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ration was not a problem because the air was kept near the saturation point. Those used for life span records were segregated by sex and maintained 5 per wide mouth jar (50 by 80 mm.). For sterility studies, individual females were placed in stender dishes (60 by 35 mm.) and each provided with an untreated male. Regular mating associated with internal fertilization was observed periodically with all pairs. Feces, debris, and dead animals were removed daily by pipette and an equal amount of fresh sea water was added. If ova or nauplii had been deposited the pair of parents were transferred by glass tube to another stender dish. All incubator Artemia were fed daily by adding dilute yeast suspension in the amount of one drop per animal to the water of the container.

Results and Conclusions

Figure 1 presents survival of Artemia after exposures to various doses of x-rays. As shown, a sex difference was revealed at higher exposures by the tendency for females to die earlier than males in simultaneous experiments.

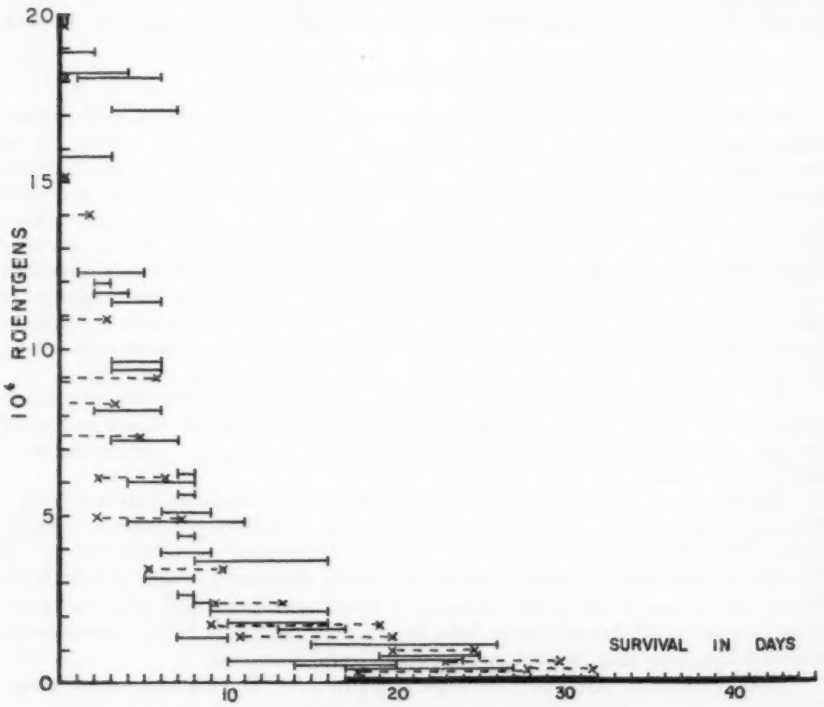

Figure 1. The range in time during which deaths in samples of five Artemia occur subsequent to x-ray exposure. Male records are designated by unbroken lines and short vertical marks; female records by broken lines ending in x marks. Control records are indicated by the thickening of the base line.

Furthermore, at 150,000 r and above, females failed to recover, although many males were able to survive for several days provided the exposure did not exceed 200,000 r. As a precaution, in the event that Artemia would be able to recove. from "knock down" doses as do adult insects, the following were delivered above those plotted: 224,910 r, 257,040 r, 289,170 r, 321,300 r, 353,430 r, and 358,785 r. No recovery occurred.

The minimal dosage at which all animals are killed seems to be the most feasible approach to lethal dose with materials of this type. As shown in Figure 1 for doses above 10,000 r, the variability in the time of death is not great when observations are taken in days for an organism whose life span is days. This is considerably different than the picture obtained with mammals whose life span is years, even decades, and the standard approach of 50% dead in 30 days provides a stable centering point for a high degree of variability. A 50% curve could be drawn for each sex through the ranges shown of Figure 1 and its shape would be

TABLE I

Summary of experiments on gamete deposit per individual female Artemia

X-ray dose in r	Highest no. of broods	Average no. of broods	Highest total no. of gametes	% Viviparous	Average no. of gametes	No. of females	Average adult life span in days
0	6	4.6	287	46.40	67.17	20	16.9
510	4	2.0	140	48.25	71.50	15	12.4
1,020	5	3.0	176	42.77	37.82	20	10.5
2,040	4	2.0	138	.04	57.46	15	9.2
3,060	2	0.9	130	.01	50.78	10	15.4
4,080	2	1.0	67	.14	29.40	10	10.7
5,160	2	0.7	55	0	18.29	15	5.9
11,527	1	0.7	79	0	31.40	5	3.4
15,900	1	0.5	23	0	6.60	5	9.8
21,200	1	0.4	51	0	12.60	5	6.6

the typical steep S. However no length of survival time suggests itself as particularly significant.

Table I presents a summary of observations on gamete production by females subsequent to their exposure. It is based upon the deposit of 46,966 ova and nauplii by 120 females. An initial experiment clearly demonstrated that no reproductive units except those pre-formed would be discharged after exposures above 5160 r and the groups of 5 experimental animals at each dose were not supplemented by replication. At 4080 r and 5160 r, exceptional individuals deposit a second meager brood but the great majority of females so treated die with empty uteri. At 3600 r second broods are adequate and death with a full uterus is not uncommon. Observations at the next lower dose, 2040 r, made it apparent that the sterility threshold had been bracketed. A comparison of the average life spans of Table I with the ranges at similar doses in Figure 1 demonstrates that deaths occurred relatively early in the sterility investigation. A number of cases of death of either or both parents following transfer lead us to suspect that brine shrimp are too delicate for repeated handling. Thus a number of females potentially able to give rise to additional broods of offspring may have been killed by

mechanical injury. Top individual performance as included in the table may be more valid as a basis for expectancy under normal conditions than are the averages obtained here. On either basis, the neighborhood of 2000 r is critical not only for numbers of broods produced but on the basis of inhibition of viviparity.

Normally, as occurred in controls, Artemia females void either live nauplii or brown heavy-shelled eggs (Lochhead and Lochhead, 1940). In addition to these, the experimentals produced several unusual types of gametes: partially formed nauplii, smooth shiny white eggs, dull white ragged eggs, orange eggs and tan eggs. Partially formed nauplii were able to complete development but none of the other types were seen to hatch. This may be due in part to their vulnerability through lack of a protective shell. Protozoa and copepods were seen actively attacking, presumably feeding on shell-less eggs.

Hatchability of brown shelled eggs will not be considered here. The data on various batches are not all identical because ways of handling this type of product were being explored.

Discussion

The lethal doses of ionizing radiation vary considerably among different types of animals. Best investigated from this standpoint have been the mammals where the total body dosage required to kill 50% of the adults within 30 days ranges from 200 to 800 r (Patt and Brues, 1954). To kill such types within hours after exposure requires higher doses, even as much as 25,000 r to 50,000 r for extreme cases (Hagen and Sacher, 1954; Patt and Brues, 1954). Massive as such doses may seem, they fall far below those producing immediate lethality for certain bacteria and protista on the one hand (Bonham *et al.*, 1947) and for adult insects on the other (Hassett and Jenkins, 1952; Sullivan and Grosch, 1953). The present results, 150,000 r for females and 200,000 r for males, place Artemia near but below holometabolous insects in radiation tolerance as adults. Demonstration of a sex difference was not unexpected. However females are usually found more resistant than males in the various animals studied.

Physiological factors undoubtedly are of importance in determining radiosensitivity and radiotolerance. One mammal, the bat, shows no effect on life span under 15,000 r just after hibernating and survives for several days after 60,000 r (Smith *et al.*, 1951). In work with insects the senior author has been impressed with the resistance of cell types specialized for physiological activity. Often this specialization is reflected cytologically in some degree of somatic polyploidy, a situation achieved through endomitosis and conceived to confer radioresistance (Grosch and Sullivan, 1954). Consistent with this view are the present findings in light of the fact that endomitosis accompanies differentiation to adult condition of a number of important Artemia tissues (Barigozzi, 1942). At earlier stages when mitosis is occurring and before endomitotic tissues have attained multiples of the basic chromosome number, Artemia should prove radiosensitive. Indeed, one of Gajewskaja's (1923) statements suggests this. Furthermore, even the most tolerant forms of holometabolous insects have radiosensitive developmental stages (for Habrobracon see Clark and Mitchell, 1952), while mammals on the other hand never achieve a radiotolerant stage. The latter depend upon mitotically active tissue to prevent loss of physiological fluids and for maintainance of the lines of bodily defence.

The most useful study found for comparison with Artemia results has been that of Henshaw (1939) on the cut branches of the colonial coelenterate, Obelia. Here 250,000 r were required to suppress hydranth activity and growth of the differentiated unit whereas only 6% of that large dose suppresses initiation and halts growth in the bud stage, a period characterized by cell proliferation. Henshaw used the same MBL generator employed for the present work.

Unlike the lethal dose response, the level of x-ray treatment causing Artemia sterilization is not appreciably above that required in some vertebrates for complete, irreversible destruction of ovaries. For example, although the 30-day lethal total-body dose in rats produces great degenerative changes in the ovaries, as much as 3000 r may be required in local exposure to insure permanent sterility (Lacassagne and Gricouroff, 1941). This may also be true for rabbits and guinea pigs. A similar dose, which as shown above affects Artemia gamete production profoundly, produces only brief temporary sterility in the wasp, Habrobracon (Grosch and Sullivan, 1954). To completely sterilize this wasp 5000 r is required. Glücksmann (1947) cites the same figure for Drosophila. Both of these holometabolous insects have a polytrophic type of ovariole in which large groups of polyploid nurse cells accompany each oocyte. Therefore on histological grounds the ovaries are not directly comparable to other forms. Evidence that insect ovaries of a simpler structure may be vulnerable at much lower doses is supplied with results with grasshopper nymphs (Tahmisian and Vogel, 1953) which have a panoistic type of ovariole. For this relatively simpler and more generally comparable tissue, 350 r is adequate to destroy all but the most advanced eggs and 800 r results in complete destruction of the ovaries. This is much like the traditional range for mammalian dosage, man and mouse, summarized by Glücksmann (1947). At present we can only speculate concerning the feature responsible for the dose requirement in Artemia sterilization. It seems likely that the interrelated shell gland may be involved. Further investigation along these lines is indicated since there is a notable lack of agreement in the literature on why reproduction is sometimes viviparous and at other times oviparous. In order to avoid mechanical damage to adults the more tedious transfer of gametes is suggested in future experiments.

Summary

As adults, Artemia males are killed by 200,000 r of x-radiation; females by 150,000 r. This approaches the doses required to kill adult holometabolous insects. Neither insects nor the brine shrimp depend upon mitotic tissues in adulthood. The sterility dose at 2000–3000 r for females is lower than in insects with polytrophic ovarioles and higher than in grasshoppers with simpler panoistic ovarioles. Further investigation is suggested for the interrelations of the Artemia shell gland and the reproductive cycle.

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LWOFFIA CILIFERA GEN. NOV., SP. NOV., A CILIATED MEMBER OF THE FAMILY SPHENOPHRYIDAE (HOLOTRICHA: THIGMOTRICHA)

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Up to the present time, three genera have been referred to the family Sphenophryidae. Two of these, *Pelecyophrya* and *Gargarius,* are monotypic. The third genus, *Sphenophrya,* includes several species, the most extensively studied one being *S. dosiniae.* An excellent summary of our knowledge of this remarkable group of ciliates has been prepared by Chatton and Lwoff (1949, 1950). Since the publication of their comprehensive monograph, one new species has been added to the genus *Sphenophrya* by Polianskiĭ (1951). A contribution of Raabe (1949), in which the development of *S. dosiniae* is compared with that of *S. sphaerii,* is apparently the only other publication dealing with this group which was not issued in time for consideration by Chatton and Lwoff.

The known genera of sphenophryids are parasitic on the gills of lamellibranch mollusks. As adults they are sessile and, with occasional exceptions in *Pelecyophrya tapetis,* are not ciliated. However, an infraciliature, disposed in two systems of a few rows each, persists in adult sphenophryids. In *P. tapetis* and the several species of *Sphenophrya,* the adults produce temporarily ciliated embryos which resemble in a general way some of the ciliates of the family Ancistrocomidae. In *Gargarius gargarius,* the embryo is also ciliated, but it is similar to the adult in all other respects by the time division has been completed. The embryonic ciliature of sphenophryids originates from the basal granules of those portions of the infraciliary rows which are conferred upon the embryo during division.

On the branchial filaments of *Brachidontes* (*Mytilus*) *recurvus* (Rafinesque) I have found a ciliate which is referable to the Sphenophryidae, but which differs from the other genera in having cilia throughout its life history. The morphology of this ciliate is most nearly like that of *Pelecyophrya,* but the process of division gives rise to two fully differentiated individuals, as is the case in *Gargarius.* The new genus is named for Dr. André Lwoff, of the Institut Pasteur, Paris, in recognition of his contributions to protozoology.

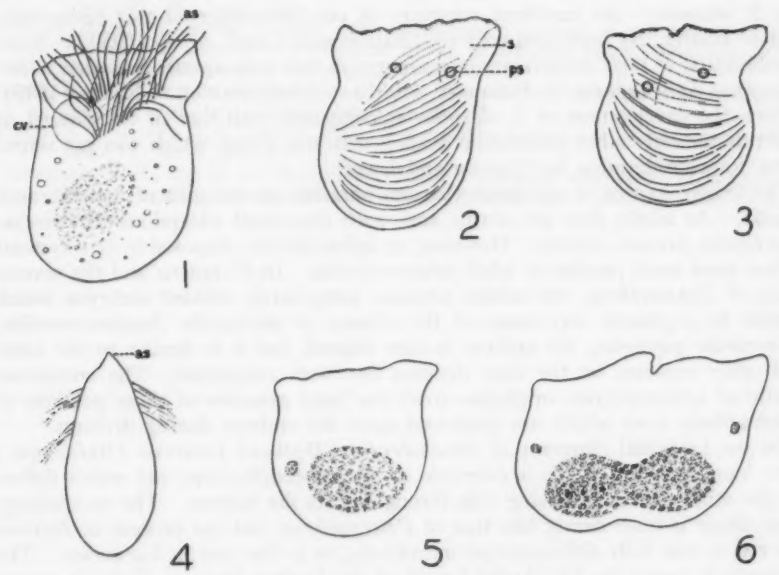
This study was carried out during the tenure of a Fellowship from the John Simon Guggenheim Memorial Foundation. It was begun while I was in residence at the Conservation Reserve of the University of Florida, and completed in the Department of Zoology, University of North Carolina.

Materials and Methods

Lwoffia cilifera was found in only one of ten specimens of *Mytilus recurvus* collected in a brackish lagoon confluent with the Caloosahatchee River approxi-

mately 6 miles southwest of Fort Myers, Florida. In the infected mussel, the ciliate occurred in enormous numbers. This permitted an extensive study of living material and the fixation of smears with hundreds of individuals.

For observation of living ciliates, small pieces of gill tissue were mounted in the fluid drained from the mantle cavity. Smears on coverglasses were fixed in Hollande's fluid with 3 per cent of acetic acid. These were stained with iron hematoxylin or the Feulgen nucleal reaction, or impregnated with activated silver albumose (Protargol) according to the method of Bodian. Impregnation with the sample of Protargol available to me was not satisfactory unless the smears were bleached, after fixation and washing, by Mallory's method, which consists of placing them in 0.5 per cent potassium permanganate for 5 minutes, washing them well, and then treating them with 5 per cent oxalic acid for 5 minutes.



EXPLANATION OF FIGURES 1–6

Lwoffia cilifera. All figures, except Figure 1, have been prepared with the aid of a camera lucida and are based on specimens fixed in Hollande's fluid and impregnated with Protargol (P) or stained by the Feulgen nucleal reaction (F); × 1640.

as, attachment surface; cv, contractile vacuole; ps, primordium of sucker; s, sucker.

FIGURE 1. From right side; after sketches from life.

FIGURE 2. From right side, showing rows of basal granules, pellicular fibril-like structures, sucker, and primordia of suckers; nuclei omitted; P.

FIGURE 3. From left side; P.

FIGURE 4. Optical section, posterior to middle; P.

FIGURE 5. Macronucleus and micronucleus; F.

FIGURE 6. Late stage of division; F.

LWOFFIA CILIFERA GEN. NOV., SP. NOV.

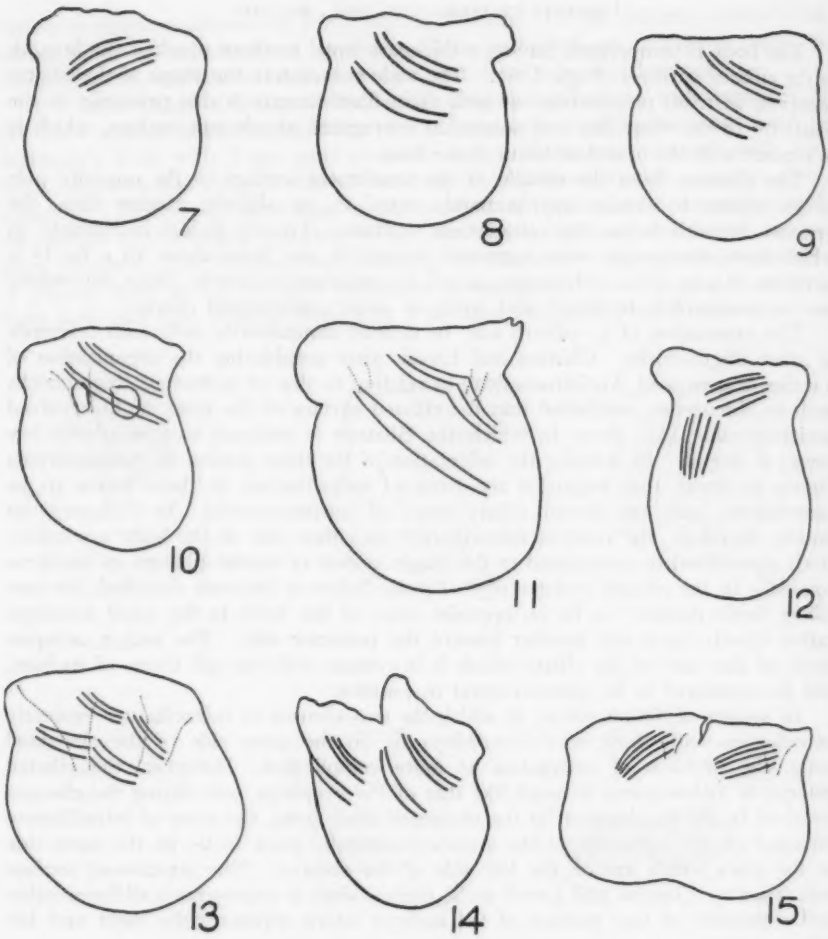
The body is compressed, having a thickness equal to about one-half the breadth of the ciliated surfaces (Figs. 1–4). The wide variation in the shape of the ciliates observed in fresh preparations as well as in fixed smears is due primarily to the plasticity of the ridge-like and somewhat corrugated attachment surface, which is in contact with the branchial tissue of the host.

The distance from the middle of the attachment surface to the opposite pole of the ciliate is usually approximately equal to, or slightly greater than, the greatest breadth below the attachment surface. Twenty living individuals in which these dimensions were measured ranged in size from about 16 μ by 14 μ to about 29 μ by 25 μ. However, as will be explained presently, these dimensions are not comparable to length and width of more conventional ciliates.

The orientation of *L. cilifera* may be defined satisfactorily only with reference to other thigmotrichs. Chatton and Lwoff, after considering the organization of a series of genera of Ancistrocomidae in relation to that of stomatous thigmotrichs such as *Ancistrum*, concluded that the ciliated surface of the more highly evolved ancistrocomids (*i.e.*, those in which the ciliature is reduced to a relatively few rows) is dorsal. In defining the orientation of the three genera of sphenophryids known to them, they regarded the rows of infraciliature in these forms to be homologous with the dorsal ciliary rows of ancistrocomids. In *Pelecyophrya tapetis*, therefore, the rows of infraciliature on either side of the body are collectively considered to correspond to the single system of dorsal ciliature in ancistrocomids. In the ciliated embryo of *P. tapetis*, before it becomes detached, the two ciliary fields destined to lie on opposite sides of the body in the adult converge rather closely upon one another toward the posterior end. The sucker occupies much of that part of the ciliate which is in contact with the gill tissue of its host, and is considered to be antero-ventral in position.

In species of *Sphenophrya*, in which the two systems of infraciliature evidently homologous with those of *Pelecyophrya* lie on the same side of the elongated body, the problem of orientation is more complicated. However, the ciliated embryo of *Sphenophrya* is much like that of *Pelecyophrya*, and during the changes involved in the development of the elongated adult form, the rows of infraciliature disposed on the right side of the embryo eventually come to lie on the same side as the rows which are on the left side of the embryo. The attachment surface was taken by Chatton and Lwoff to be dorsal, since it represents a differentiation and expansion of that portion of the embryo which separates the right and left ciliary systems.

The attachment surface of *Lwoffia* is more extensive than that of *Pelecyophrya*, and is disposed on those portions of the body which correspond to the anterior end and anterior part of the dorsal surface in *Pelecyophrya*. The sucker of *Lwoffia* resembles that of *Pelecyophrya* in having a broad funnel-shaped outline when viewed from the right or left side of the body (Figs. 2, 3). The margins of the sucker may be discerned in some specimens stained with iron hematoxylin, and individuals impregnated with Protargol show them quite regularly. The sucker becomes broader antero-dorsally, and its anterior margin may often be traced as far as the attachment surface. I have been unable to determine how much of the attachment surface is occupied by the origin of the sucker, and I have



EXPLANATION OF FIGURES 7–15

Lwoffia cilifera. All figures have been prepared with the aid of a camera lucida and are based on specimens fixed in Hollande's fluid and impregnated with Protargol; × 1640. The rows of cilia are rendered diagrammatically as continuous lines.

FIGURE 7. From right side.

FIGURES 8, 9. From left side.

FIGURE 10. Enlargement of primordia of suckers.

FIGURE 11. Elongation and early stages of division of ciliary rows.

FIGURES 12–14. Later stages in separation of ciliary systems of prospective daughter ciliates.

FIGURE 15. Attachment surfaces of prospective daughter ciliates distinct.

not observed an actual orifice. The sucker is evidently a protoplasmic channel. Although the cytoplasm of *L. cilifera* contains abundant lipoid granules, stainable with Sudan III in fresh preparations, and smaller, relatively non-refractile granules, cell fragments from host tissue cannot be identified. It appears probable that nutrition of the ciliate involves absorption of relatively fluid materials.

In impregnated specimens there is almost invariably observed within the sucker a small sphere whose periphery is argyrophilic (Figs. 2, 3). Another sphere of this type is characteristically located posterior to the region occupied by the sucker. These two spheres are primordia of new suckers formed during division of the cilate, and may therefore be regarded as homologues of the sucker primordia observed by Chatton and Lwoff in about 30 per cent of the individuals of *P. tapetis* stained with iron hematoxylin. Apparently, however, neither of the two primordia in *P. tapetis* lies within the sucker of the parent ciliate.

The cilia are approximately 8 μ or 9 μ long. After being detached from the gill tissue of its host, *L. cilifera* is capable of swimming actively, although its movements are largely erratic rotations.

In proportion to the size of the body, the ciliary rows are much shorter than the rows of infraciliature in *P. tapetis*. In the latter species, some of the rows extend almost the full length of the ciliate. On the right side of the body of *L. cilifera* there are five rows arranged in one group (Figs. 1, 2, 7). On the left side of the body, there are two groups of rows (Figs. 3, 8, 9), as in *Pelecyophrya*. The group which is nearer to the attachment surface consists of three rows in all suitably oriented impregnated specimens which I have studied. The group which is farther from the attachment surface consists most frequently of two rows, but individuals with three rows in this group are common. In two specimens, out of hundreds I studied, there were four rows in this group, and in one specimen there was a single row.

In Protargol preparations, a number of delicate fibril-like structures, which appear to be pellicular, are observed on the right and left sides of the body, in both the ciliated and unciliated portions (Figs. 2, 3). Many of these, at least, traverse the entire broad surface, and at either end there is a convergence of the fibril-like structures from the right and left sides.[1]

The large macronucleus is located in the postero-ventral half of the body. It is elongated, and is usually about one and a half to two times as long as it is wide. In life, the macronucleus is coarsely granular (Fig. 1), with some of the granules being more or less rod-shaped. After fixation it is stained so heavily by the Feulgen nucleal reaction and by iron hematoxylin that its granular character is often obscured. The micronucleus generally lies close to the macronucleus. In the interphase it is typically spherical, with the chromatin dispersed in rather fine granules (Fig. 5). The contractile vacuole is located in the posterior part of the dorsal half of the body.

Stages of division are abundant in my stained and impregnated preparations of *L. cilifera*. In impregnated specimens, in which nuclear changes cannot be followed satisfactorily, the earlier stages of fission are evidenced by the division of each row of cilia into two segments. The two daughter segments are generally

[1] Comparable configurations are prominent in my Protargol preparations of *Gargarius gargarius* and *Sphenophrya dosiniae*.

approximately equal (Figs. 12–14), and it appears that the parent row elongates considerably before the break in the continuity of the row occurs (Fig. 11). As the furrow of cytoplasmic cleavage becomes more pronounced, the systems of ciliary rows formed by division of the parent system on either side of the body become more widely separated, and the attachment surfaces of the two prospective daughter ciliates become quite distinct (Figs. 14, 15).

During the early stages of division, the primordia of the new suckers to be conferred upon the daughter ciliates enlarge. Evidently this enlargement may be initiated before there is a break in the continuity of any of the ciliary rows (Fig. 10). Since one of the primordia lies within the sucker of the parent ciliate, the new sucker derived from this primordium essentially replaces the old sucker in the same position. As the development of new suckers progresses, the argyrophilic material of the primordia loses prominence.

Division of the micronucleus apparently begins after the primordia have begun to enlarge, and is completed before the attachment surfaces of the prospective daughter ciliates may be recognized as separate. Macronuclear division coincides with later stages of cytoplasmic division (Fig. 6).

Lwoffia gen. nov.

Diagnosis: Sphenophryid ciliates with cilia persisting throughout the life history. The body is laterally compressed, with an extensive ridge-like attachment surface which occupies the anterior end and anterior part of the dorsal surface of the body. The ciliature of the right side is composed of one group of rows; the ciliature of the left side is arranged in two groups of rows. The sucker is funnel-shaped in outline as viewed from the side, and is directed antero-dorsally; it is considered to be a protoplasmic channel. Primordia of new suckers to be formed during division are evident in the interphase, and one of the primordia lies within the sucker of the parent ciliate. Genotype: *Lwoffia cilifera.*

Lwoffia cilifera sp. nov.

Diagnosis: Size approximately 16 μ by 14 μ to 29 μ by 25 μ. The ciliary system of the right side of the body is composed of 5 rows. On the left side of the body, the ciliary rows in the group nearer the attachment surface are three in number, and the rows in the group farther from the attachment surface are commonly two or three in number, rarely one or four. On the branchial filaments of *Brachidontes recurvus* (Rafinesque), near Fort Myers, Florida. Syntype slides are in the collection of the author.

Summary

To the existing genera of sphenophryid ciliates (*Sphenophrya, Pelecyophrya,* and *Gargarius*) described by Chatton and Lwoff, a new genus, *Lwoffia,* is added. The genotype, *L. cilifera,* was found on the gills of *Brachidontes recurvus* near Fort Myers, Florida. Functional cilia persist throughout the life history of *L. cilifera,* and are disposed in two systems on either side of the body, in much the same way as the rows of infraciliature in *Pelecyophrya tapetis.* The attachment surface is proportionately more extensive than that of *P. tapetis,* and in form is much like that of species of *Sphenophrya.* Division produces equal and fully-

differentiated ciliates, rather than an ancistrocomid-like embryo from the parent ciliate, and in this respect is similar to division in *Gargarius gargarius*.

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THE SIZE, SHAPE AND HYDRATION OF LOBSTER HEMOCYANIN[1]

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Sedimentation (Eriksson-Quensel and Svedberg, 1936), diffusion (Polson, 1939a), partial specific volume (Svedberg, 1939), intrinsic viscosity (Polson, 1939b), and sedimentation equilibrium (Eriksson-Quensel and Svedberg, 1936) studies have been carried out on the hemocyanin of the lobster, *Homarus vulgaris*. While these studies serve in a general way to characterize the hemocyanin molecule, several important pieces of information are lacking. First, sedimentation coefficients were not extrapolated to zero concentration. Second, no information concerning hydration was provided. Third, no independent evidence of shape was presented. The purpose of the present study is to provide a more precise and more complete characterization of the lobster hemocyanin molecule.

A. Preparation of Hemocyanin

North American lobsters, *Homarus americanus*, obtained in Woods Hole, Massachusetts, were used in this study. The technical descriptions of the species appearing in Milne-Edwards (Milne-Edwards, 1837) permit the conclusion that *H. americanus* and *H. vulgaris* are very similar if not actually indistinguishable. The lobsters were bled, the blood was defibrinated by whipping, and the hemocyanin was purified by alternate centrifugation for ten minutes in a Servall angle centrifuge at 5000 rpm and for six hours in a type 40 rotor in a Spinco Model L centrifuge at 35,000 rpm. All centrifugations were carried out in the cold. Two low speed and one high speed runs were used to prepare the hemocyanin for diffusion and sedimentation experiments. Several additional cycles of low and high speed centrifugation were used in the preparation of material for partial specific volume, intrinsic viscosity, and specific refractive increment measurements.

B. Sedimentation Studies

1. Sedimentation as a function of concentration

Sedimentation studies on various concentrations of hemocyanin in $M/10$ phosphate buffer at pH 7 were carried out in a Spinco ultracentrifuge. Solution densities were measured with a pycnometer. Hemocyanin concentrations were determined by means of a refractometer, using 0.00193 as the specific refractive increment. Solution viscosities were measured at a temperature below and at a temperature above that of the sedimentation run, and the value at the tempera-

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[2] Publication no. 32 of the Department of Biophysics.

TABLE I

Sedimentation of lobster hemocyanin at various concentrations

Hemocyanin concentration (g/100 ml.)	s Svedbergs	η Solution viscosity (cps)	ηs	ηs corrected	η_0 Solvent viscosity (cps)	$\eta_0 s$	$\eta_0 s$ corrected	Solution density
10.7	7.65	2.1454	16.4	20.1	1.0439	8.0	9.8	1.053
4.77	14.1	1.4008	19.8	23.0	1.0305	14.6	16.9	1.039
2.75	18.0	1.2179	21.9	24.5	1.0362	18.7	20.9	1.029
1.55	20.1	1.0982	22.0	24.1	1.0057	20.2	22.1	1.024
1.5	20.9	1.0156	21.2	23.2	0.9000	18.8	20.6	1.024
0.5	22.8	0.9859	22.5	24.3	0.9200	21.0	22.7	1.020
0.0				24.5			24.5	

ture of the sedimentation run was determined by interpolation. Solvent viscosities were determined in the same way. The product of sedimentation coefficient and solution viscosity and the product of sedimentation coefficient and solvent viscosity were then each corrected for solution density by multiplying by

$$(1 - V_h' d_w^{20}) / (1 - V_h' d),$$

where V_h' has the meaning defined in the next section, d_w^{20} is the density of water at 20°, and d is the density of the solution investigated in the ultracentrifuge. The figures thus obtained are identified by the symbols ηs (corr) and $\eta_0 s$ (corr), respectively. The data are listed in Table I.

It was found that viscosity-sedimentation products gave more nearly linear plots with concentration than did reciprocals of viscosity-sedimentation products. In the case of most virus characteristic particles, solution viscosity-sedimentation products are essentially invariant with concentration (Lauffer, 1944; Lauffer and Stanley, 1944; Taylor, Epstein and Lauffer, 1955). This was not found to be the case with lobster hemocyanin. Solution viscosity-sedimentation products, and solvent viscosity-sedimentation products were extrapolated to the same value at zero concentration. This value is listed in Table I. When one divides it by the viscosity of water at 20°, 0.01005, one obtains the sedimentation coefficient, corrected to water at 20°, at infinite dilution. This has a value of 24.5×10^{-13} cgs units. The previously reported value, 22.6×10^{-13} (Eriksson-Quensel and Sved-

TABLE II

Sedimentation of lobster hemocyanin in sucrose solutions

s Svedbergs	η Solution viscosity (cps)	ηs	d Solution density
18.85	1.158	21.8	1.0314
11.9	1.555	18.5	1.0587
7.0	2.196	15.4	1.1027
3.28	3.727	12.2	1.1445
1.05	8.287	8.7	1.1909
0.22	19.793	4.4	1.2420
0.06	25.463	1.4	1.2719

berg, 1936) is the mean of the values obtained from various dilutions of lobster blood in different electrolyte solutions; it does not represent the value at infinite dilution.

2. Sedimentation in sucrose solutions of various densities

Sedimentation studies on 0.9% hemocyanin in 0.1 *M* phosphate buffer at pH 7, to which various amounts of sucrose had been added, were carried out. Sedimentation coefficients, solution viscosities and solution densities were evaluated as described previously. The data are recorded in Table II. The product of solution viscosity and sedimentation coefficient, not corrected in any way, was plotted against solution density. The data fit the straight line, $\eta s \times 10^{15} = 104.7 - 80.9d$. The intercept at a density of 0.998, the density of water at 20°, is 24.0×10^{-15}. This is in good agreement with the solution viscosity-sedimentation coefficient product for 0.9% hemocyanin solution, 24.2×10^{-15}, as interpolated from the data of Table I. The intercept corresponding to zero sedimentation rate occurs at a

TABLE III

Viscosity at various concentrations

Concentration (g/ml.) C	Concentration (volume fraction) $\phi = CV$	Relative viscosity η_r	$\frac{\eta_r - 1}{\phi}$
0.107	0.0792	1.9881	12.5
0.0477	0.0353	1.3259	9.25
0.0275	0.0204	1.1448	7.1
0.0155	0.0116	1.0713	6.15
0.0083	0.0062	1.0300	4.85

density value of 1.294. The reciprocal of this value, 0.773, is taken to be the effective specific volume of the hydrated hemocyanin molecule, V'_h (Lauffer, Taylor and Wunder, 1952).

C. Diffusion Experiments

The diffusion coefficient of an 0.9% hemocyanin solution in 0.1 *M* phosphate buffer at pH 7 was determined at 20° in a Perkin-Elmer electrophoresis apparatus. A value not significantly different from that reported by Polson (Polson, 1939a) was obtained. Because Polson's data are more extensive, his value for D_w^{20} of 2.77×10^{-7} cm.²/sec. will be used in subsequent calculations.

D. Intrinsic Viscosity

Viscosities of solutions of various concentrations of hemocyanin in 0.1 *M* phosphate buffer at pH 7 were determined using a capillary viscometer in a water bath at 25°. Concentrations were measured by means of a refractometer using a value for specific refractive increment of 0.00193. The data are shown in Table III. Intrinsic viscosities or viscosity numbers were evaluated by plotting the quotient of relative viscosity increment divided by volume fraction against

volume fraction and extrapolating to zero. A value of 4.85 ml. per ml. was obtained. Polson (Polson, 1939b) reported a value of 6.4. The value obtained in the present study, namely 4.85, is used in subsequent calculations.

E. Partial Specific Volume

The apparent partial specific volume of lobster hemocyanin was calculated from the dry weight composition and the density of an aqueous solution of a highly purified preparation. A mean value not significantly different from that for the partial specific volume reported by Svedberg (Svedberg, 1939), namely 0.74, was obtained.

Figure 1. Electron micrograph of hemocyanin molecules. Uranium shadowed (× 69,000). Shadow angle slope − ¼. Line corresponds to one micron.

F. Specific Refractive Increment

The specific refractive increment was determined on aqueous solutions of highly purified hemocyanin by means of a Phoenix Precision differential refractometer. The concentration was determined by dry weight. The specific refractive increment obtained was 0.00193. Redfield had previously reported a value of 0.002 (Redfield, 1934).

G. Electron Microscopy

Electron micrographs of hemocyanin molecules shadowed with uranium were kindly made by Dr. Irwin Bendet of the Department of Biophysics at the Uni-

versity of Pittsburgh. A representative micrograph is shown in Figure 1. The electron micrograph shows clearly that the hemocyanin molecules are not rod-shaped. The shadow lengths are consistent with a height of 7.5 mμ and the modal image size corresponds to a particle with a diameter of 33 mμ. Thus, the micrograph is consistent with the assumption that the hemocyanin molecule is a flattened ellipsoid of revolution about 33 mμ in diameter and 7.5 mμ thick. Naturally, these dimensions refer to dried particles.

Discussion

The following parameters, determined in the present and in previous investigations, can be used to calculate the size, shape, and degree of hydration of lobster hemocyanin; sedimentation coefficient, $s_w^{20} = 24.5 \times 10^{-13}$; diffusion coefficient, $D_w^{20} = 2.77 \times 10^{-7}$; partial specific volume, $V = 0.74$; effective specific volume of the hydrated particle, $V_h' = 0.773$; intrinsic viscosity or viscosity number, $[\eta] = 4.85$. Details of these calculations have been reviewed recently (Lauffer and Bendet, 1954).

From the sedimentation coefficient, the diffusion coefficient, and the partial specific volume, the molecular weight and the friction ratio can be calculated to be 825,000 and 1.25, respectively. This molecular weight value supersedes the value of 752,000 reported by Eriksson-Quensel and Svedberg (Eriksson-Quensel and Svedberg, 1936) and compares with their sedimentation equilibrium value of 803,000. From the partial specific volume, V, and the effective specific volume of the hydrated particle, V_h', one can calculate that hemocyanin is hydrated to the extent of 0.15 ml. of water per g of anhydrous protein. The reservations which must attend the use of hydration values calculated in this manner have been discussed previously (Lauffer, Taylor and Wunder, 1952; Lauffer and Bendet, 1954).

If it is assumed that the molecule is a flattened ellipsoid of revolution and that its hydration is actually equal to the value calculated from sedimentation vs. medium density studies, namely, 0.15 ml. of water per g protein, then one can interpret the friction ratio, 1.25, to be consistent with a hydrated flattened ellipsoid of revolution with an axial ratio of 4.14, corresponding to a diameter of 32.2×10^{-7} cm. and a thickness of 7.8×10^{-7} cm. These values are consistent with those estimated from the electron micrograph. The axial ratio of the hydrated particle calculated on the basis of the same assumptions from the intrinsic viscosity was 4.05, in good agreement with the value obtained from sedimentation, diffusion and partial specific volume data.

Summary

1. The sedimentation coefficient of lobster hemocyanin corrected to water at 20° and extrapolated to zero concentration has been found to be 24.5×10^{-13} cgs units.

2. The intrinsic viscosity has been found to be 4.85 ml./ml.

3. From the variation of the sedimentation rate of hemocyanin in sucrose solutions of different densities and from the partial specific volume, the hydration of hemocyanin was calculated to be 0.15 ml. of water per gram of dry protein.

4. The specific refractive increment was determined to be 0.00193 ml. per 10^{-2} g.

5. From the above parameters and others taken from the literature, lobster hemocyanin molecules were calculated to have a molecular weight of 825,000, corresponding to oblate ellipsoids of revolution with hydrated diameter of 32.2 mμ and hydrated thickness of 7.8 mμ.

6. Electron micrographs are consistent with the assumption that the molecules are plate-like ellipsoids of revolution 33 mμ in diameter and 7.5 mμ in thickness in the dried state.

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STUDIES ON THE NEUROSECRETORY SYSTEM OF IPHITA LIMBATA STAL. I. DISTRIBUTION AND STRUCTURE OF THE NEUROSECRETORY CELLS OF THE NERVE RING

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Neurosecretory cells have been discovered by several authors in different groups of insects (see reviews: Scharrer and Scharrer, 1954a, 1954b). In *Iphita limbata* (Pyrrhocoridae: Hemiptera) the presence of these cells in the brain and the metathoracic ganglion was reported by Nayar (1953) who also described certain changes in the activity of these cells correlated with reproduction in the female. The functions of the neurosecretory cells of the brain have been worked out in certain insects, especially by Scharrer (1952) in *Leucophaea maderae*, by Thomsen (1952) in *Calliphora erythrocephala*, and by Williams (1952) in *Platysamia cecropia*. The present series deals with a more detailed study of the structure, functions and development of the neurosecretory cells of *Iphita limbata* Stal.

Materials and Methods

Adult insects were used for the study. They were frequently collected fresh from the field and were kept in insectary boxes where they were fed on cotton seeds.

When the dorsal wall of the cranium is removed and the head is stretched forwards by pulling the rostrum and fixing it with plasticine, the brain becomes exposed. The dissection of the brain was done under a stereoscopic binocular microscope (magnification $\times 40$). A longitudinal tracheal tube with a number of tracheoles traverses the middle of the cerebral ganglia. When that is removed, faintly whitish spots become visible underneath the firm and thin membrane investing the brain. When this membrane is teased with a fine needle, two groups of medial neurosecretory cells come into view as bluish-white masses on either side of the midline. Each contains about sixteen cells. The medial neurosecretory cells can be removed as a group from the pars intercerebralis of the brain with fine forceps (*cf.* Thomsen, 1952). When observed in insect Ringer, these remain without marked changes for about an hour.

For the study of topography and histological structure of the neurosecretory cells, the entire nerve ring was removed and fixed. The medial neurosecretory cells of the pars intercerebralis of the female were selected for the examination of finer cytological details; the corresponding male tissue shows no marked difference in cellular structure and distribution.

The following methods were used in this study:

1. For general histology: Bouin's, Helly's, Smith's, and Baker's formal-calcium were used as fixatives. Staining was done in Heidenhain's iron hematoxylin, Masson's trichrome (Foote, 1933), Gomori's chrome alum-hematoxylin-phloxin (Gomori, 1941), Gomori's aldehyde fuchsin (Pearse, 1953) and Heidenhain's Azan (Pantin, 1948).

2. For supravital observation: Phase-contrast and dark field microscopes. Light microscope for supravitally stained tissue (neutral red, methylene blue, and dahlia violet) in 0.001% stain for 10 to 15 minutes.
3. For the study of the granular system in the cytoplasm: Material fixed in Baker's ISO fixative (osmic acid in sucrose-iodate solution) stained in Altmann's acid fuchsin according to Metzner's method, and Helly-fixed material stained in Hirschler's hematoxylin (Baker, 1951).
4. For the study of the spheroidal system in the cytoplasm: Classical Golgi methods, such as fixation in Flemming-without-acetic and staining in iron hematoxylin; Weigl's Mann-Kopsch; Kolatchew's and Aoyama's methods (Baker, 1951); Thomas' (1948) method of study of gradual osmification in 2% osmium tetroxide; Baker's (1949) technique of sudan black staining; and Thomas' (1948) method of sudan black staining for paraffin sections.
5. For other structural details: Unna-Pappenheim's methyl green-pyronin method (Darlington and LaCour, 1947) after fixation in Heidenhain's saline-mercuric chloride, for nucleic acids; Baker's acid hematein test (1946) and pyridine extraction test for phospholipines; Nath's (1934) method of staining fats by Sudan III; Barnett and Bourne's (1942) method for ascorbic acid; treatment with Millon's reagent after Bouin-fixation, xanthoproteic reaction, Pollister's method after Bouin-fixation, Hartig-Zacharias' method after formal-calcium fixation, for proteins (Pearse, 1953); Best's carmine after Bouin-fixation for glycogen (Pearse, 1953); indole reaction, Vulpian reaction, Sevki's Giemsa-tannin method and Lison's chromaffin test (Pearse, 1953); and Schmorl's method for lipofuscins (Pearse, 1953).

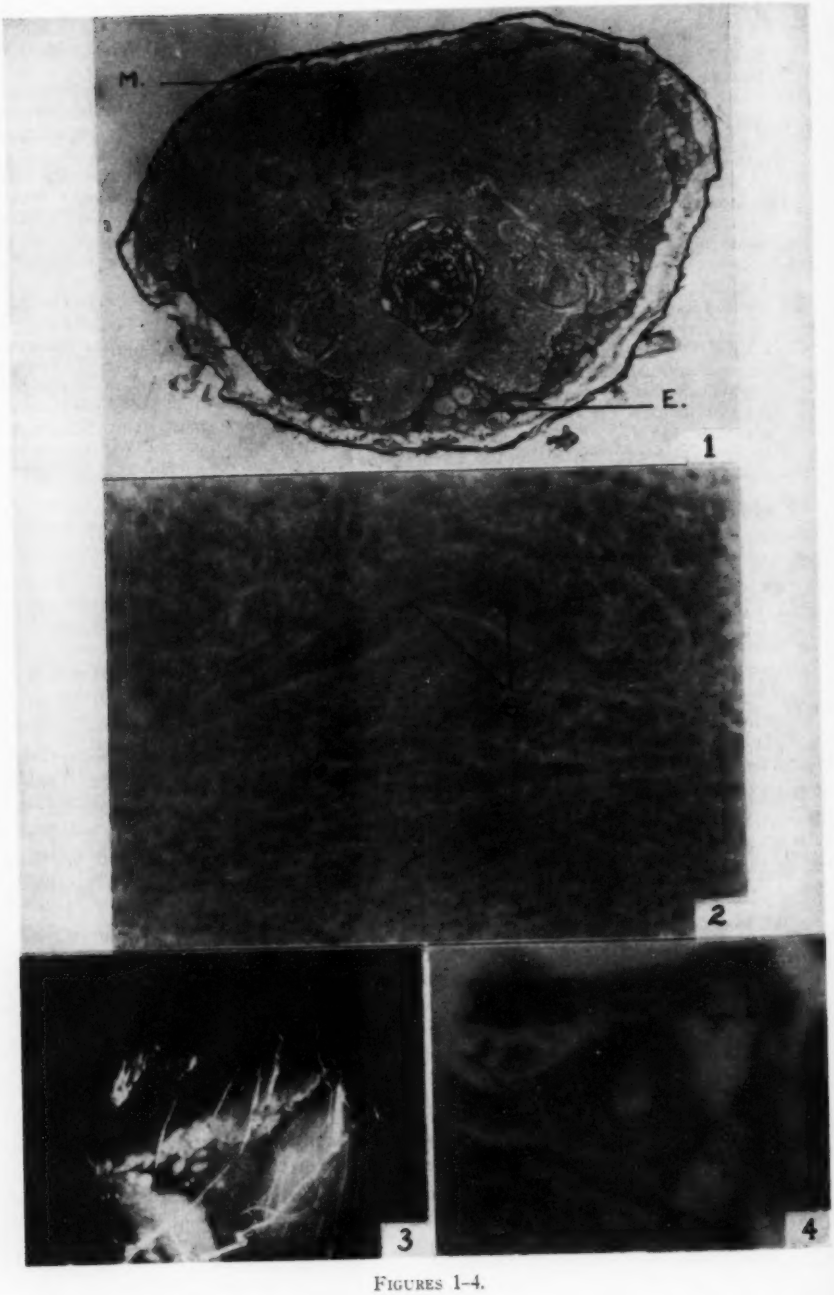
Fixed material was processed according to Peterfi's double embedding method with one-half to one per cent celloidin in methyl benzoate, and paraffin sections were cut at 5 μ for general staining. Some thick setions, 6 to 8 μ, were also cut for the study of the spheroidal constituents of the cells, while for mitochondria sections 2 to 3 μ were used. Frozen sections were cut at 10 and 15 μ after embedding in gelatine (25 per cent gelatine with trace of cresol).

Observations

For histological details fixation in Bouin's and Smith's fluids followed by Gomori's chrome alum-hematoxylin-phloxin gave the best results. The chrome hematoxylin selectively stains the neurosecretory cells a deep blue; sometimes in thick sections (6 or 8 μ) the cytoplasm appears blackish blue. Equally good results were obtained by using Azan stain where the cytoplasm is colored brilliant red by the azocarmine. The cytoplasm of these cells is fuchsinophilic in Masson's stain.

The neurosecretory cells are distributed in different parts of the nerve ring. In addition to the median neurosecretory cells of the brain (pars intercerebralis), there are the lateral groups of neurosecretory cells of the protocerebrum, numbering about three or four on each side. They are much smaller than the medial cells and rarely appear bluish in the fresh brain. The subesophageal ganglion contains scattered neurosecretory cells laterally and ventrally along the margin of the neuropile (Figs. 1, 5).

The neurosecretory cells of *Iphita* show two types of response to the staining procedures used. In one type, the cytoplasmic inclusions are stained deep blue in chrome hematoxylin-phloxin and dark red in Azan; these cells may be designated as "A cells." In the other type, the cytoplasmic contents stain red in chrome hematoxylin-phloxin and light blue in Azan; these may be designated as "B cells."



FIGURES 1–4.

In Heidenhain's iron hematoxylin, the "A cells" are colored bright blue, and the "B cells" light blue. No selective staining of any kind was obtained by Gomori's aldehyde fuchsin.

The distribution of "A cells" and "B cells" is characteristic. In all preparations, the majority of the medial neurosecretory cells of the protocerebrum belong to the "A type" while a few (varying in number from two to six) are similar to "B type" cells. The latter show in their cytoplasm scattered granules. It is possible that these cells may be "A cells" deprived of the bulk of secretory material. Under certain experimental conditions where the insects have been fed on salt water or where salt water has been injected into the hemocoele, all the cells of the medial clusters are colored blue. The lateral neuroscretory cells and most of the subesophageal cells belong to the "B type" with phloxinophilic cytoplasm. A few cells of the subesophageal mass show a resemblance to the "A type." A map showing the distribution of these cells in the ring is given in Figure 6.

Lying on the two sides of, and closely apposed to, the anterior end of the aorta are the tiny corpora cardiaca with a slender bridge-like mass of cells in between which represents the hypocerebral ganglion. The cells of the hypocerebral ganglion are phloxinophilic, resembling the "B type" cells of the nerve ring. Laterally are the compact corpora cardiaca which show in their cytoplasm, in the vicinity of their nerves, granules colored blue in chrome hematoxylin. The cytoplasm here is heterogeneous, unlike that of the cells of the hypocerebral ganglion. The few cytoplasmic granules in the corpora cardiaca resemble those in the "A type" neurosecretory cells.

The medial cerebral neurosecretory cells have been used for a more detailed study. They measure about 52 to 97 μ in length. The cell tapers towards the axon and the apical part is swollen and carries the eccentrically located nucleus. The broadest part measures 32 to 39 μ. The round nucleus is 13 μ in diameter.

If pressure is exerted on a fresh preparation, within a few minutes globule-like droplets, measuring up to 13 μ, often separate off from the abaxonal part of the cell.

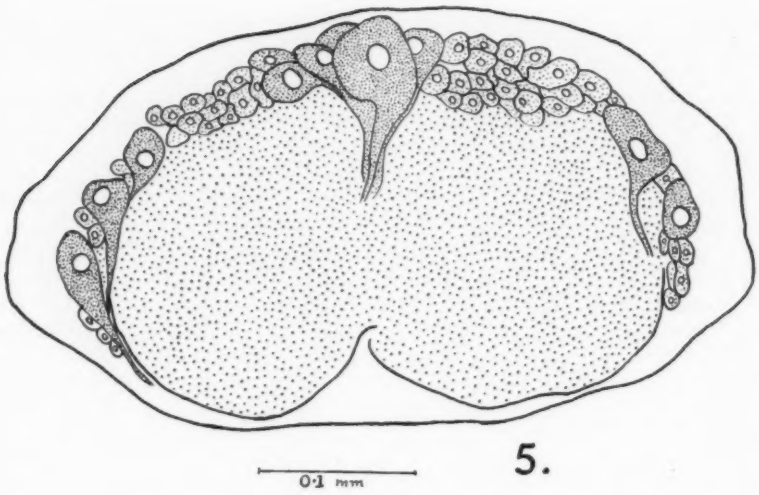
Under the phase contrast microscope, the cell is seen to contain a transparent and eccentric nucleus of low refractive index (Fig. 2). The chromocenters in the nucleus and the nucleolus appear dark, with a higher refractive index. The cytoplasm is filled with dark masses of granules which have generally a clumped appearance. Towards the broader edge of the cell are numerous tiny, dark granules which exhibit very active Brownian movement. The axons are traceable up to nearly three times the length of the cell. In the axons, as well as in the cytoplasm, are spheroids (see below) of variable size, with clear, dark rims and transparent

FIGURE 1. Transverse section of the nerve ring of *Iphita limbata* passing through the medial neurosecretory cells of the brain and the subesophageal ganglion. In the center is the esophagus. M = medial cells of the pars intercerebralis some of which are stained blue in Gomori's chrome-hematoxylin-phloxin. E = esophageal neurosecretory cells. Approx. × 95.

FIGURE 2. A medial neurosecretory cell of the pars intercerebralis under the phase contrast microscope. A few spheroids (S) are in focus. Approx. × 700.

FIGURE 3. Medial neurosecretory cells under the dark field microscope. They appear white; darker bodies in the cells are the nuclei. The white streaks are the tracheae. Approx. × 60.

FIGURE 4. The spheroids of the neurosecretory cell stained black in hematoxylin after fixation in Flemming without acetic acid. Approx. × 750.



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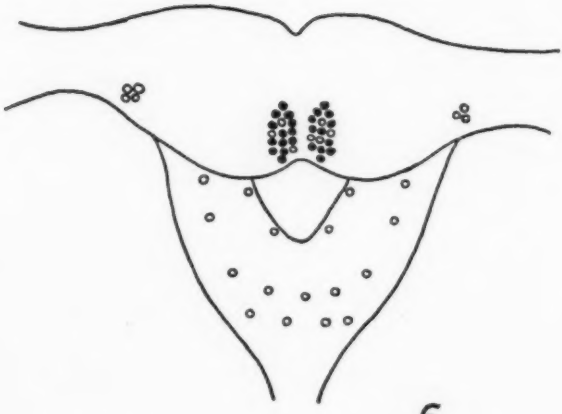

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FIGURE 5. Camera lucida drawing (composite from a few adjacent sections) showing the marginal distribution of the subesophageal neurosecretory cells.

FIGURE 6. Diagram showing the distribution of the neurosecretory cells in the nerve ring of *Iphita limbata*. "A type cells" shown as black dots; "B type cells" shown as circles.

interior, appearing as extremely tiny droplets. Dark patches of a blotchy nature are seen on the nuclear membrane. The spheroids could be made out only with difficulty, but they could easily be distinguished when the distribution had been made out previously by vital staining methods.

In the dark field microscope the cytoplasmic content of the cell looks shiny and bluish-white, in the form of granules (Fig. 3). Besides granules there are larger bodies which are probably spheroids. The granules seem to flow along the axons.

Supravital staining in 0.001% neutral red gave good and uniform pictures of the cytoplasmic content. The cytoplasm shows red spheroids of variable size (Fig. 7). The neutral red spheroids measure from 0.71 μ to 2.86 μ in diameter. Somewhat similar results have been obtained by staining in 0.001% methylene blue. The spheroids here appear blue but the general staining effect is not quite as good as in neutral red.

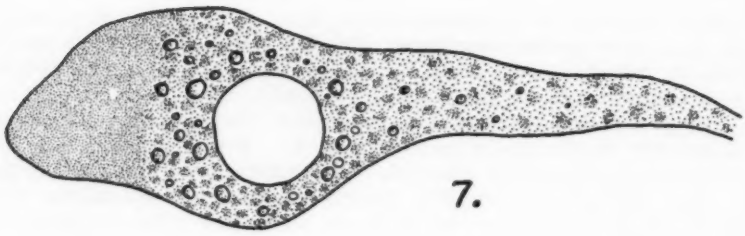
Supravital staining in 0.001% dahlia violet also gives a satisfactory picture of the cytoplasmic structures. The entire cell shows a very faint violet tint. The granulated mass stands out as dark greyish-violet bodies performing active movements. These granules show a blotchy appearance due to clumping. The spheroids do not always show up well; sometimes they appear as rounded bodies with dark violet rims and clear interior. Dahlia violet staining is not quite as good as neutral red; but in good preparations the granules and spheroids are traceable as conspicuously colored materials into the axons also.

The abaxonal broad part of the cell contains a vacuole-like structure which enlarges in due course into a conspicuous watery vacuole. Dense masses of granules fill this vacuole which show continual Brownian movement (Fig. 7). In the course of time these vacuoles part from the cells and appear as transparent drops filled with colored granules. These droplets sometimes take a faint reddish tint in neutral red. Such vacuoles have a low refractive index. The granules stain in the same way as mitochondria with Metzner's and Hirschler's methods. It is possible that these granules are derived from mitochondria, but this cannot be demonstrated conclusively with the methods used in this investigation.

Thus the study of living cells under phase-contrast and after supravital staining reveals that the cytoplasmic content of the medial neurosecretory cells of the brain is a complex of two substances: (1) a granular mass of small bodies, stainable dark greyish-violet by dahlia violet supravitally, contained in a fluid-filled vacuole; (2) a spheroidal system of tiny vacuole-like bodies, variable in size, stainable by neutral red, dahlia violet, and somewhat poorly by methylene blue.

An important structure in the cytoplasm of the neurosecretory cell is the spheroidal system. It consists of vacuole-like tiny spheres of variable size, supravitally stainable by neutral red, methylene blue, and dahlia violet.

The spheroids are demonstrable by the classical "Golgi" methods. When the cells are fixed in Flemming-without-acetic and stained in Heidenhain's iron hematoxylin, the spheroids appear as black bodies (Fig. 4). The spheroids are osmiophilic, and many of them appear as definite rings, while others look like large granules in Mann-Kopsch preparations after impregnation with osmic acid for two and a half days. These granules of different sizes could be seen on the nuclear membrane also. Similar bodies are discernible when cells are treated for about three days according to Kolatchew's method. The method of Aoyama is excellent to demonstrate the "Golgi system." The cytoplasm contains a system



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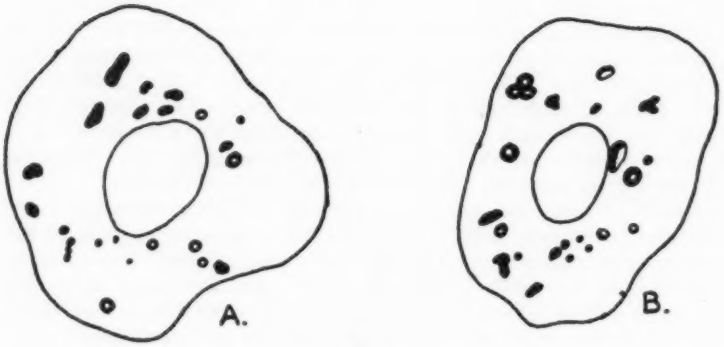
10μ.



10μ.

8.

FIGURE 7. Camera lucida drawing of a medial neurosecretory cell of the brain, stained in 0.001% neutral red. The neutral red vacuoles forming the spheroids are shown as circles. At the abaxonal part of the cell is a vacuole filled with mitochondria (fine stipples). The mitochondria of the rest of the cell appear in groups.

FIGURE 8. Camera lucida drawings of: A. A medial neurosecretory cell after treatment according to Aoyama's method, showing the "Golgi apparatus." The small spheroids which have coalesced appear as irregular masses, while the larger ones are ring-shaped. B. A neurosecretory cell after treatment according to Baker's sudan black method. Note similarity of the spheroids in both preparations.

of spheroids which show a deposit of silver around the periphery of the large spheroids, while the smaller vacuoles are more or less completely blackened. The picture in Aoyama preparations closely resembles the cells stained by neutral red; the neutral red spheroidal structures corresponding to the black ones in silver preparations (Fig. 8A).

Thomas' technique is very useful for the study of the development of the osmiophilic structures in the spheroids of the live cells. Freshly dissected neurosecretory cells were placed on a slide in a drop of 2% osmic acid. The coverslip was then sealed off. Within about five minutes the cells appear brownish. This becomes well marked in about thirty minutes; after about fifty minutes a few fine black granules and crescent-shaped black rims make their appearance. These are the developing osmiophilic elements. The subsequent deposition of osmium is comparatively slow. By about sixteen hours, the spheroids look like rings with the periphery almost completely blackened. In the deeper parts of the cell, such complete ring-like formation occurs in about a day. At room temperature (28–29° C.) the spheroids of all sizes become completely blackened after the fifth day. The cytoplasm as a whole then gets tinged with dark grey.

This method showed the gradual blackening of the margin of the spheroids which ultimately produced the configuration seen in the classical "Golgi preparations."

Baker's sudan black method shows that these spheroids are lipoidal in constitution. This is further supported by the acid hematein test which gives a positive result. Sudan black staining in both frozen and paraffin sections has similar results, and the preparations strikingly resemble those described above (Fig. 8B).

The spheroidal system of the neurosecretory cells could be reasonably described as lipochondria of variable size, characteristically osmiophilic, argentophilic, and sudanophilic.

In addition to the secretory granules and the spheroid system, other constituents of the neurosecretory cells were examined. In sections fixed in Heidenhain's mercuric-saline, and stained with methyl green-pyronin according to a modification of Unna-Pappenheim, the cytoplasm of the neurosecretory cells showed red or dark pink coloration indicating a concentration of ribose nucleic acid. The large nuclei of these cells are colored light pink, having in some cases a faint greenish tinge also, which indicates a comparatively low concentration of chromosome nucleic acid.

Baker's acid hematein test showed that the medial neurosecretory cells of the brain react strongly positively. The cytoplasmic products are colored a brilliant blue in both A and B types of cells. The mitochondrial and lipochondrial materials of the cytoplasm react like this: Pyridine extraction followed by acid hematein test shows no coloration at all. This is positive indication of the presence of phospholipines in the cytoplasm.

Though sudan black selectively stains the lipochondria found in the spheroids, simple staining by Sudan III according to Nath's method was unsuccessful. There was no indication of any coloring in these cases.

Barnett and Bourne's method for vitamin C revealed the presence of scattered black granules in the cytoplasm of the neurosecretory cells. They are more numerous in a perinuclear zone and close to the nuclear membrane.

Best's carmine test for glycogen on Bouin-fixed material was negative in the cytoplasm of the neurosecretory cells.

An indication of the presence of protein material in the cytoplasm was observed. A pink or light brick-red color developed after treatment with Millon's reagent for one hour at 60° C. after Bouin-fixation. There was no definite result with the xanthoproteic reaction. Sections of brain fixed in formal-calcium and treated with potassium ferrocyanide and ferric chloride showed an especially bright blue color in the cytoplasm of the neurosecretory cells, indicating the presence of protein. Bouin-fixed material treated according to Pollister's method also revealed the presence of a high protein content in these cells (development of a brick-red color in contrast to the light color of other parts of the brain).

Repeated tests were made to determine whether chromaffin granules are present in the cytoplasm of the neurosecretory cells. The chromaffin test of Lison, indole reaction, Vulpain reaction and Sevki's Giemsa staining method all gave negative results.

In cytochemical studies, Gomori's chrome-hematoxylin-phloxin method has been described to be selective for lipofuscins. The deep blue neurosecretory material of *Iphita limbata* may be considered as lipofuscins. But Schmorl's method for melanin and lipofuscin is not very useful for the characterization of neurosecretory cells.

Discussion

The distribution of the neurosecretory cells of the brain resembles that reported in other groups of higher insects. Those of the hemipteran brain have been described by Hanström (1938). The presence of neurosecretory cells has also been reported in the subesophageal ganglion, which forms an important neurosecretory center in orthopteroid insects (Scharrer, 1941). In an earlier note (Nayar, 1953) neurosecretory cells in the brain of *Iphita limbata* were described which stain blue with Gomori's chrome-hematoxylin-phloxin. In the present study, two types of neurosecretory cells are described in this species, A and B cells, which can be distinguished by their staining properties.

From a study of histological sections and from experimental investigations, various authors (Scharrer and Scharrer, 1944; Scharrer, 1952; E. Thomsen, 1952, 1954; Arvy, Bounhiol and Gabe, 1953; M. Thomsen, 1954) concluded that the neurosecretory products are transported along the axons and, in the case of the protocerebral neurosecretory cells, reach the corpus cardiacum, where they are stored. The observations in *Iphita limbata* support this view, the neurosecretory material being traceable along the nervi corporis cardiaci.

E. and M. Thomsen (1954) described the appearance of living neurosecretory cells in the darkfield microscope. The fresh cells of Iphita studied in ordinary, darkfield, and phase contrast microscopes show signs of a pronounced glandular activity. The large nucleus resembles that of other glandular cells with conspicuous nucleolus and chromonemata with large chromocenters. The cytoplasm is densely filled with secretory material which is seen to flow along the axons. This product resembles that observed in the corpora cardiaca of *Locusta migratoria* (Nayar, 1954).

The spheroids are osmiophilic and argentophilic and so give rise to the classical

"Golgi" pictures. A similar spheroidal system has been described in *Locusta migratoria* (in the metathoracic motor neurons by Shafiq, 1953, and in the corpus cardiacum by Nayar, 1954). Baker (1950) has pointed out that spherical or spheroidal bodies in live cells are of a lipoidal nature; the smaller ones are lipoidal throughout and the others contain a spherical vacuole of non-lipoid material within, so that the lipoid is in the form of an enveloping sheath or externum. This description also applies to the spheroids seen in the neurosecretory cells of *Iphita limbata*.

The entire content of the cytoplasm of the median neurosecretory cells of the brain is rich in phospholipines. This is evidenced by the positive bright blue coloration with acid hematein and by the lack of coloration with acid hematein after extraction with pyridine. Baker (1946) has pointed out that mitochondria in many cases react positively to the test, and Cain (1947) has shown that the lipochondria contain phospholipines. The phospholipines of the neurosecretory cells may be the combined lipines of the spheroidal and mitochondrial substances.

Part of the cytoplasmic content is ascorbic acid appearing as black granules when the cells are subjected to treatment according to the method of Barnett and Bourne (1942). These authors have described the presence of vitamin C granules in the neurons of the chick. The distribution in the neurosecretory cells of *Iphita limbata* is somewhat similar with granules scattered in the cytoplasm and apposed to the nuclear membrane.

The different tests for proteins, precipitin, etc. have indicated the presence of some type of protein in the cytoplasm. Tests for chromaffin inclusions and glycogen gave negative results. Cameron (1953) has suggested that the chromaffin content of the corpus cardiacum in the locust is secreted by the gland itself and is not elaborated by the neurosecretory cells. This may also apply to *Iphita limbata*, because chromaffin material is not seen in the cytoplasmic content of these cells.

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Summary

1. In *Iphita limbata* (Pyrrhocoridae: Hemiptera) the brain contains paired medial and lateral groups of neurosecretory cells, and the subesophageal ganglion scattered marginal neurosecretory cells. The medial cells (pars intercerebralis) number about 16 on each side, the lateral three or four.

2. On the basis of their staining properties two types of neurosecretory cells can be distinguished in the nerve ring of Iphita. "A cells" staining a deep blue with Gomori's chrome-hematoxylin-phloxin and a bright red with Azan make up most of the neurosecretory cells of the brain. The subesophageal ganglion contains "B cells," staining red with Gomori's method and light blue with Azan.

3. The corpora cardiaca and their nerves (nervi corporis cardiaci) contain a material similar in its staining properties to that of the "A cells."

4. The medial neurosecretory cells of the protocerebrum contain large nuclei with a low, and nucleoli with a higher, refractive index. The nucleoli are phlox-

inophilic and azocarminophilic. The cytoplasm contains granules and spheroids. The granules appear black in the phase contrast microscope, violet with dahlia and, like mitochondria, red with acid fuchsin; they exhibit continual Brownian movement in the living cells. The granules are associated with a fluid material. The spheroidal system which is osmiophilic, argentophilic, and sudanophilic, represents the lipochondria. Both the granular and spheroidal systems are revealed by supravital staining methods.

5. The cytoplasm of the neurosecretory cells contains a high concentration of ribose nucleic acid. The nuclear membrane and the cytoplasm show granular concretions of vitamin C. The secretory material contains proteins as indicated by various tests. The granular and spheroidal constituents are rich in phospholipines.

6. Tests for chromaffin substances and glycogen gave negative results. Staining methods for lipofuscins show that the product in the "A cells" probably contains these very complex substances.

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THE EFFECT OF X-RADIATION ON ENZYME SYSTEMS OF TETRAHYMENA PYRIFORMIS[1, 2]

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It had been shown previously by Eichel and Roth (1953b) that x-radiation at a level of 300,000 or 500,000 r depressed the respiration of *Tetrahymena pyriformis* W,[3] but did not appreciably affect the nuclease activity in homogenates prepared from irradiated whole cells. In irradiated homogenates, however, nuclease activity was reduced by approximately 50%. This paper presents some further observations of the effect of 300,000 to 600,000 r on respiration and several enzymes of *T. pyriformis* S. The S strain was chosen for two reasons; first, to obtain comparisons of the effect of x-radiation on respiration and deoxyribonuclease (DNase) activity in a strain different from that studied previously, and second, because the higher rate of succinate oxidation in the S strain (Eichel, 1954) would facilitate investigations of this specific enzyme system. The effects of x-radiation on catalase, and malic, glutamic, and succinic dehydrogenases, as well as some properties of succinic dehydrogenase and DNase, have also been observed. In addition, the effect of x-radiation on the oxidation of L-phenylalanine and acetate by the W strain has been studied.

Materials and Methods

T. pyriformis S cells were grown and harvested as previously described (Eichel and Roth, 1953b), and homogenates were prepared as described by Eichel (1954). Measurements of respiration and oxidative enzyme activities were by conventional Warburg techniques; the details of the procedures for x-irradiation and for the respiration and DNase studies were similar to those previously reported (Eichel and Roth, 1953b). Measurements of DNase activity were made at regular intervals for at least 100 to 136 minutes. The components of the test systems used in the study of oxidative enzyme activities are given in connection with the tables of results. All Warburg runs were made at 27° with air as the gas phase and a fluid volume of 3.0 ml. Catalase was determined by the method of Bonnichsen, Chance and Theorell (1947). Unless otherwise stated, 72 hour cultures of strain S were utilized for all experiments.

[1] Work performed under Contract No. AT(30-1)-1069 for the Atomic Energy Commission.

[2] A preliminary account of this project was presented at the General Scientific Meetings, Marine Biological Laboratory, Woods Hole, Mass., in August 1953 (Eichel and Roth, 1953a).

[3] *T. pyriformis* was formerly called *T. geleii.*

Experimental Results

The effect of x-radiation on respiration

Concentrated cell suspensions (approximately 2×10^6 cells/ml.), and cell suspensions diluted with an equal volume of glass-distilled water (referred to as diluted cell suspensions), were irradiated. The appearance of the irradiated cells immediately after exposure was similar to that of the x-irradiated W strain; many cells were non-motile, swollen, and highly vacuolated. The effect on respiration was comparable to that observed with the W strain (Eichel and Roth, 1953b) with the exception that the S organisms appeared more resistant at the 300,000 r level. It is evident from the data in Table I that 600,000 r was considerably more damaging than 300,000 r, and that at the higher level there was little difference in

Table I

Effect of x-irradiation with 300,000 and 600,000 r on respiration of T. pyriformis *S*
The figures give the average and range

Conditions	Per cent change in O_2 uptake from control cells			QO_2 of control cells[2] (μl O_2 per hr. per mg. of dry wt.)
	Minutes after irradiation[1]			
	30	80	140	
Concentrated cell suspension (7)[3]	+12	−3	−9[4]	32.3
300,000 r	(+3 to +17)	(0.0 to −7)	(−6 to −13)	(30.8 to 39.8)
Diluted cell suspension (6)	−4	−17	−23	29.9
300,000 r	(+40 to −43)	(0.0 to −47)	(−8 to −48)	(25.3 to 34.0)
Concentrated cell suspension (3)	−16	−40	−50	27.7
600,000 r	(+8 to −40)	(−22 to −52)	(−37 to −60)	(21.1 to 32.0)
Diluted cell suspension (6)	−5	−40	−54	27.8
600,000 r	(+35 to −38)	(−20 to −55)	(−40 to −70)	(26.7 to 29.5)
Part 2. Effect of 2,6-diaminopurine (AP)				
Diluted cell suspension+AP[5] (2)[6]	+13	−24	−41	
300,000 r	(0, +26)	(−17, −31)	(−36, −46)	
Diluted cell suspension (2)[7]	+31	−3	−9	
300,000 r	(+22, +40)	(0, −5)	(−8, −10)	
Diluted cell suspension+AP[5] (5)[6]	+23	−25	−41	
600,000 r	(+60 to −4)	(−9 to −45)	(−26 to −60)	
Diluted cell suspension (4)[7]	−8	−41	−57	
600,000 r	(+35 to −22)	(−20 to −48)	(−37 to −63)	

[1] The elapsed time between the end of irradiation and closing of the stopcocks was 20 minutes. Therefore, the first 10 minute reading was actually made 30 minutes after irradiation. In general, readings were made at 10-minute intervals during the first hour and at the end of 2 hours. For the sake of brevity, portions of these data are presented here.

[2] Calculated for the first hour.

[3] Numbers in parentheses indicate the number of separate experiments in both Part 1 and Part 2.

[4] Six determinations; two at 110 minutes, four at 120 minutes.

[5] AP concentration was 3.3×10^{-2} *M* during irradiation and 6×10^{-3} *M* after addition of aliquots to vessels.

[6] Control was unirradiated diluted cell suspension+AP.

[7] Control was unirradiated diluted cell suspension.

effect using concentrated or diluted cell suspensions. At 300,000 r, however, the concentrated cell suspensions showed less depression of respiration than those that were diluted. The results at 30 minutes after exposure to the x-ray beam were extremely variable except with concentrated suspensions irradiated with 300,000 r. The initial increase in respiration under these conditions is of interest; it is probably somewhat greater than the data indicate since staining (dilute methyl green) of the irradiated cells showed that many of them (25–50%) were dead immediately after irradiation.[4] In general, there was a significant decrease in respiration of the x-irradiated cells with time; this may be due to a continuing mortality of damaged organisms. The QO_2 values of control cells are presented in Table I to indicate the level of activity in these experiments.

A general interest in the purine antagonist, 2,6-diaminopurine (AP), led us to study the effect on respiration of the presence of this compound during irradiation of whole cells. Controls consisted of unirradiated cells in the presence of similar concentrations of the antagonist. The results obtained with AP are given in Table I, part 2. It can be seen that this compound afforded appreciable protection at the higher level of exposure, particularly in the early post-irradiation period. On the other hand, at 300,000 r AP had a significant deleterious effect. The concentration of AP used (0.006 *M*) was slightly inhibitory to the respiration of control cells. At the present time it is not possible for us to offer an explanation of the effects observed. With regard to the protective action, several amino compounds have been shown to protect other x-irradiated animals (Bacq and Herve, 1951; Bacq *et al.*, 1953), and it is possible that AP is another member of this class.

The effect of x-radiation on the oxidation of L-phenylalanine and acetate

Of the common amino acids, *L*-phenylalanine is most rapidly oxidized by *T. pyriformis* W (Roth, Eichel and Ginter, 1954). The rate of oxidation of acetate is also high in this organism (Kidder and Dewey, 1949). In view of the stimulatory effect of these compounds on the respiration of whole cells, it was deemed of interest to determine what effect they would have when added to x-irradiated cells. Strain W was used since it was investigated previously with respect to the oxidation of these metabolites. Diluted cell suspensions were prepared and then irradiated with 300,000 r. Immediately afterward, aliquots of the suspensions were added to Warburg vessels whose sidearms contained either neutral *L*-phenylalanine or acetate solution. Oxygen consumption was measured after tipping the sidearms and was compared to unirradiated controls to which the same additions had been made, as well as to controls, both irradiated and non-irradiated, to which only distilled water had been added from the sidearms. Experimental details and results are given in Table II. It can be seen that the oxygen consumption in the presence of *L*-phenylalanine was inhibited by 43% (average of 4 experiments) 30 minutes after irradiation. This inhibition was observed to increase at a uniform rate until, at 140 minutes post-irradiation, the value was 58%. The decrease in oxygen uptake of irradiated cells to which phenylalanine had been added was somewhat greater than that observed for the irradiated cells without phenylalanine, studied at the same time. The conclusion is that x-irradiation impairs the ability

[4] Living cells, even those visibly severely damaged, do not take up the stain; dead cells stain a light green with a darker green nucleus.

TABLE II

Effect of x-irradiation with 300,000 r on oxidation of L-phenylalanine and acetate by T. pyriformis. *Each vessel contained the following: body, 1.0 ml. of either control or irradiated cells and 1.0 ml. of 0.1 M Na_2HPO_4-NaH_2PO_4 buffer, pH 7.3; sidearm, substrate as indicated below or water; center well, 0.2 ml. of 20% KOH. The vessels were allowed to equilibrate 5 minutes before tipping in substrate. After an additional 5 minutes, the stopcocks were closed and readings were taken at 10-minute intervals during the first hour and at the end of 2 hours*

Additions from sidearm	Conditions and strain	O_2 uptake in μl.					
		Minutes after closing stopcocks[1]					
		10	% change	60	% change	120	% change
L-phenylalanine[2]	(unirradiated) W(5)[3]	31		202		430	
L-phenylalanine	(irradiated)	24	−23	146	−28	249	−42
Acetate[4]	(unirradiated)	43		270			
Acetate	(irradiated)	51	+19	250	−7		
Water	(unirradiated)	30		182		354	
Water	(irradiated)	26	−13	143	−21	251	−29
L-phenylalanine	(unirradiated) S(3)	29		179		336	
L-phenylalanine	(irradiated)	15	−48	86	−52	142	−58
Acetate	(unirradiated)	31		177		337	
Acetate	(irradiated)	36	+16	167	−6	260	−23
Water	(unirradiated)	32		157		275	
Water	(irradiated)	18	−43	84	−47	131	−48
L-phenylalanine	(unirradiated) W(2)	17		148		301	
L-phenylalanine	(irradiated)	9	−47	64	−57	101	−66
Acetate	(unirradiated)	25		158		283	
Acetate	(irradiated)	20	−20	125	−21	170	−40
Water	(unirradiated)	14		104		203	
Water	(irradiated)	6	−57	47	−55	81	−60
L-phenylalanine	(unirradiated) W(2)	29		188		356	
L-phenylalanine	(irradiated)	14	−52	80	−57	129	−64
Acetate	(unirradiated)	35		184		341	
Acetate	(irradiated)	33	−6	153	−17	227	−33
Water	(unirradiated)	21		125		240	
Water	(irradiated)	16	−24	89	−29	154	−36
Averages							
L-phenylalanine			−43		−49		−58
Acetate			+ 2		−13		−32
Water			−35		−38		−43

[1] See note 1, Table I.
[2] Final concentration = 3×10^{-3} *M*.
[3] Numbers in parentheses indicate age of culture in days.
[4] Final concentration = 5×10^{-2} *M*.

of the cells to oxidize *L*-phenylalanine. In several cases acetate stimulated oxygen consumption by irradiated cells at 30 minutes after exposure; at 80 minutes after exposure, oxygen uptake was inhibited by only 13% as compared to the 38% inhibition of endogenous respiration. Although the inhibition of acetate oxidation increased considerably between 30 and 140 minutes after irradiation, the final level attained was less than that of either the endogenous respiration or phenylalanine oxidation.

The marked ability of acetate to stimulate the oxygen uptake of irradiated cells is of considerable interest. For example, in the third experiment at 80 minutes after exposure, irradiated cells to which acetate had been added showed an increase of 170% in oxygen uptake compared to irradiated cells without acetate. In the same experiment, acetate increased the oxygen consumption of control cells

TABLE III

Succinic dehydrogenase activity of x-irradiated homogenates and of homogenates prepared from x-irradiated cells of T. pyriformis *S. Each vessel contained the following: body, 1.0 ml. of either control or irradiated homogenate, 1.0 ml. of 0.1 M Na_2HPO_4-NaH_2PO_4 buffer, pH 7.3, 0.3 ml. of 0.004 M $CaCl_2$ + $AlCl_3$, and 0.2 ml. of distilled water; sidearm, 0.3 ml. of 0.5 M sodium succinate adjusted to pH 7.4; center well, 0.2 ml. of 20% KOH. Since it has been shown that under the conditions employed here added cytochrome c has little effect on the activity of this system (Eichel, 1954), the compound was omitted. Conditions of equilibration and reading the same as those described in Table II. The figures give the average and range*

Conditions	Dose	Per cent change in O_2 uptake from control homogenates		
		Minutes after irradiation		
	(r)	30	80	140
Irradiated homogenates	300,000 (4)*	−28 (+5 to −45)	−34 (−14 to −48)	−27 (−4 to −50)
	600,000 (4)		−41 (−30 to −53)	−38 (−24 to −49)
Homogenates of irradiated cells	300,000 (4)	−15 (0 to −25)	−10 (−7 to −15)	−6 (+4 to −12)

* Numbers in parentheses indicate the number of separate experiments.

by only 52%. The net effect of the increased oxidation of acetate by irradiated cells is to give the appearance of a protective action by acetate, but in this case the acetate has been added after irradiation and the only protection would be that related to delayed effects of radiation. While certain unknown and probably complex factors will protect animals when given after irradiation (Kelly and Jones, 1953; Cole and Ellis, 1954), no simple substances have yet been shown to have this effect, and it may be worthwhile to test acetate for this action in higher animals.

The effect of x-radiation on respiratory enzymes

Succinic dehydrogenase

Homogenates were irradiated; also, whole cells were irradiated and then homogenized. In all cases suitable unexposed controls were prepared and as-

sayed simultaneously with the irradiated samples. The contents of the Warburg vessels and other experimental details are presented in Table III which gives the results obtained. Irradiation of homogenates at 300,000 or 600,000 r resulted in the loss of a considerable fraction of the activity. However, the activity of homogenates prepared from cells irradiated at 300,000 r declined only slightly, the decrease being just on the borderline of significance.[5]

Seaman (1952), using cystine as an inhibitor, concluded that *Tetrahymena* succinic dehydrogenase is a sulfhydryl-dependent enzyme. In Table IV are listed some effects of SH-reagents on the succinic dehydrogenase activity of normal homogenates. The strong inhibition of the system by *p*-chloromercuribenzoate (CMB) and *o*-iodosobenzoate (IOB) suggests further that it is SH-dependent, although the activity was not restored by the addition of glutathione at concentrations 10–20 times those of the inhibitors. The glutathione was either present in the main body of the vessels at the start of the experiment or tipped in from the sidearm midway through the run. The very slight reduction in succinic dehydrogenase activity resulting from the irradiation of whole cells is of interest in view of the studies of Barron (1946) which indicated that inactivation of SH-enzymes is an important aspect of radiation damage in animal tissues. Previously, it had been shown that the activity of the SH-dependent ribonuclease of *T. pyriformis* W was relatively unchanged after exposing cells to very high doses of x-rays (Eichel and Roth, 1953b). These observations with protozoan enzymes are consistent with the recent reports that the activities of SH-dependent enzymes are frequently not altered in the tissues of mammals exposed to high levels of x- or γ-radiation (LeMay, 1951; Roth *et al.*, 1952; Thomson *et al.*, 1952; Ashwell and Hickman, 1952; Roth *et al.*, 1953).

TABLE IV

Effect of p-chloromercuribenzoate (CMB) and o-iodosobenzoate (IOB) on succinic dehydrogenase activity of homogenates of T. pyriformis *S. Assays were performed as described in Table III except that in one experiment succinate was added to the body instead of the sidearm together with the sulfhydryl reagents. Concentrations given for the latter are final. The figures represent the average of 2 experiments*

Addition	Per cent inhibition
0.9×10^{-3} *M* CMB	−98
0.1×10^{-3} *M* CMB	−84
0.1×10^{-2} *M* IOB	−94
0.5×10^{-3} *M* IOB	−83

Glutamic and malic dehydrogenases

The conditions and results are given in Table V. The activities of both enzymes were significantly reduced at 80 minutes; after an additional hour, the inhibition of glutamic dehydrogenase was apparently decreased while there was little further change in malic dehydrogenase.

[5] Changes of less than 10% are not considered significant.

TABLE V

Glutamic and malic dehydrogenase activities of T. pyriformis *S homogenates x-irradiated with 600,000 r. Both enzymes were assayed by the method of Seaman (1951) except that each vessel contained 0.5 ml. of 0.1 M Na_2HPO_4-NaH_2PO_4 buffer, pH 7.3. Conditions of equilibration and reading the same as those described in Table II. The figures give the average and range*

Dehydrogenase	Per cent change in O_2 uptake from control homogenates	
	Minutes after irradiation	
	80	140
Glutamic (3)*	−28 (−14 to −40)	−10 (−9 to −10)
Malic (4)	−40 (−9 to −65)	−41 (−21 to −60)

* Numbers in parentheses indicate the number of separate experiments.

Catalase

T. pyriformis S contains an active and, under the conditions employed here, relatively unstable catalase which has not been previously described. No loss of enzyme activity was observed in homogenates kept at 0° up to 2½ hours. However, after 24 hours at this temperature, the activity decreased by 50%. In preliminary experiments, the activity was found to be proportional to the quantity of homogenate used. Catalase was significantly inhibited in x-rayed homogenates tested 5 minutes after irradiation (Table VI), but the activity was rapidly recovered when these homogenates were allowed to stand at 0° in the absence of substrate. In two experiments in which homogenates were irradiated at 600,000 r (not tabulated), and in which catalase assays were performed at 5, 35, 65, 95 and 155 minutes after irradiation, the average changes from control values were −36, −9, −10, −1 and +2%, respectively. After 24 hours, the irradiated homogenates were 29% more active than the controls. A similar recovery of activity was observed in homogenates prepared from irradiated cells (not tabulated). In one experiment with the latter, the catalase activity was 29% less than the control 10 minutes after irradiation, while 120 minutes later, it was 10% more active than the unchanged control.

TABLE VI

Catalase activity of x-irradiated homogenates of T. pyriformis *S. Each determination performed with 1.0 ml. of homogenate. Measurements made within 5 minutes after completion of irradiation*

Dose (r)	Specific activity × 1000		Change (%)
	Control (per sec. per mg. dry wt.)	X-ray (per sec. per mg. dry wt.)	
300,000 (2)*	2.14 (1.99, 2.29)	1.85 (1.99, 1.72)	−13 (0, −25)
600,000 (5)	2.36 (2.10 to 2.77)	1.67 (1.12 to 2.27)	−29 (−11 to −50)

* Numbers in parentheses indicate the number of separate experiments.

TABLE VII

Effect of x-irradiation with 500,000 r, and effect of sulfhydryl reagents, on DNase activity of homogenates of T. pyriformis *S. In part 1, experiments were performed as previously described for ribonuclease (Eichel and Roth, pp. 355–56, 1953b). Homogenates were irradiated in the presence of p-chloromercuribenzoate and aliquots were used for DNase assays. Before assaying, p-chloromercuribenzoate, in the concentration listed below, was in contact with the homogenate for 100 minutes, the time required for the irradiation procedure. The addition of aliquots of these samples to the assay mixtures diluted the p-chloromercuribenzoate to 3.5 × 10^{-4} M. The cysteine was added after irradiation in a final concentration of 3.3 × 10^{-3} M. In parts 2 and 3, the final concentrations are listed. The figures give the average and range of three experiments in part 1 and two experiments each in parts 2 and 3*

Part	Experimental conditions	Specific activity × 100 (units[1] per mg. dry wt.)	Change (%)
1	Control	1.38 (1.22 to 1.60)	
	Irradiated	0.66 (0.46 to 0.90)	−57 (−44 to −72)
	Control + 2.8 × 10^{-3} *M* CMB	1.20 (0.86 to 1.47)	−15 (+11 to −30)
	Irradiated + 2.8 × 10^{-3} *M* CMB	0.19 (0.13 to 0.29)	−86 (−78 to −94)
	Irradiated + 2.8 × 10^{-3} *M* CMB + 3.3 × 10^{-3} *M* L-cysteine	0.27 (0.07 to 0.55)	−82 (−58 to −99)
2	Control	1.23 (1.09, 1.36)	
	Control + 2.2 × 10^{-4} *M* CMB	1.09 (1.02, 1.16)	−8 (−6, −10)
	Control + 1.7 × 10^{-3} *M* L-cysteine	0.84 (0.79, 0.89)	−30 (−28, −31)
	Control + 2.2 × 10^{-4} *M* CMB + 1.7 × 10^{-3} *M* L-cysteine	1.04 (0.98, 1.11)	−11 (−8, −14)
	Control + 1.1 × 10^{-3} *M* IOB[2]	1.28 (1.13, 1.44)	+8 (+4, +11)
3	Control	0.76 (0.80, 0.72)	
	Control + 5.6 × 10^{-5} *M* CMB	0.87 (1.00, 0.74)	+19 (+26, +3)
	Control + 2.8 × 10^{-4} *M* CMB	0.73 (0.84, 0.63)	−4 (+6, −13)
	Control + 5.6 × 10^{-4} *M* CMB	0.72 (0.72, 0.72)	−5 (−10, 0)
	Control + 1.1 × 10^{-3} *M* CMB	0.34 (0.32, 0.35)	−55 (−60, −51)

[1] One unit of activity is defined as that amount of enzyme which, in 25 ml. of test solution, causes a decrease in optical density of 0.1 in 60 minutes.

[2] *o*-Iodosobenzoate.

The effect of x-radiation and sulfhydryl reagents on DNase activity

It has been reported previously that DNase activity decreased 50% in *T. pyriformis* W homogenates irradiated at 500,000 r (Eichel and Roth, 1953b). Similar results were obtained with the S strain and these are given in Table VII, part 1. The DNase activity of control homogenates was of the same magnitude as that found previously for the W strain. The effect of CMB, both alone and in com-

bination with x-radiation, was also investigated. The combined inhibitory effects of radiation and the presence of CMB were somewhat more than additive, while the addition of cysteine after irradiation had little effect on the inhibition. In a similar experiment with ribonuclease of the W strain, cysteine added to irradiated homogenates completely reversed the inhibition due to CMB (Eichel and Roth, 1953b). While cysteine (Table VII, part 2) inhibited DNase by 30% and CMB depressed the activity by 8%, together the two compounds produced only an 11% inhibition. CMB alone had a variable effect depending on its concentration (Table VII, part 3). At the highest concentration used, DNase activity was depressed strongly, while at the lowest concentration there was a slight acceleration. This variable response to CMB has also been reported with pancreatic ribonuclease (Ledoux, 1953). IOB accelerated DNase activity slightly. These facts suggest that in *T. pyriformis* S DNase is dependent for optimum activity on a certain ratio of -S-S- to -SH groups.

Discussion

From the results reported in this paper and the previous one (Eichel and Roth, 1953b) it seems clear that irradiation of *Tetrahymena* homogenates at 300,000 to 600,000 r results in appreciable destruction of enzyme activity. However, in the case of catalase, where irradiated homogenates were allowed to stand at 0° and assayed at varying intervals, all of the activity was recovered within 1–2 hours after x-raying. On the other hand, the irradiation of whole cell suspensions, while causing considerable decrease in respiration, does not markedly affect the activity of several enzymes tested. It seems probable, therefore, that the explanation of the high cell mortality observed does not lie in alterations in enzyme activity due to the direct or indirect action of radiation. Other factors, such as denaturation of nucleoprotein resulting in lethal mutations, or formation of toxic products *in vivo*, may be involved. Further biochemical studies on radiation effects might be more profitably directed toward changes in nuclear activities, but before this can be done we must learn more about the fundamental properties of the nucleus.

Summary

1. The effect of 300,000 to 600,000 r on respiration and oxidative and other enzymes of *Tetrahymena pyriformis* S has been determined. Both homogenates and whole cells were irradiated. At 600,000 r, respiration was significantly decreased; the activities of the succinic, glutamic, and malic dehydrogenase systems, catalase, and DNase were depressed in homogenates irradiated at this level. Within a short time after irradiation, catalase activity was completely recovered in homogenates allowed to stand at 0°. Irradiated cells showed only minor changes in succinic dehydrogenase activity.

2. Whole cells irradiated with 600,000 r in the presence of 2,6-diaminopurine were considerably protected from the effects of radiation on respiration for a short time after exposure. Cells of the W strain, irradiated with 300,000 r and then placed in the presence of *L*-phenylalanine, showed a loss of ability to oxidize this compound compared to controls. Under the same conditions acetate stimulated the oxygen consumption of x-irradiated cells to a much greater degree than that of control cells when measured 30 minutes after irradiation. Some effects of

sulfhydryl reactants on succinic dehydrogenase and deoxyribonuclease activities of homogenates were studied, and the general implications of the results were discussed.

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EFFECTS OF VARIOUS LEVELS OF X-IRRADIATION ON THE GAMETES AND EARLY EMBRYOS OF FUNDULUS HETEROCLITUS [1]

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Fish gametes and embryos have not been studied very extensively in radiobiology. This may be due to the fact that these cold blooded forms appear to be rather radioresistant and experiments must therefore be conducted at high levels of exposure. Nevertheless, the abundance and availability of such marine material and the transparency of the egg and the developing embryo make the fish an attractive object for radiobiological research. In this study an attempt is made to determine (1) the levels of radiation sensitivity of the egg, sperm, and early cleavage stages and (2) the effect of x-irradiation upon early development of the embryo of *Fundulus heteroclitus*.

Materials and Method

Adult specimens of this fish were brought to the laboratory immediately after being caught, and the sexes were separated into different 15-gallon tanks through which ran fresh sea water. Since such fish in captivity generally release their gametes within a day or so, they were used immediately and discarded.

Eggs and sperm were obtained by the usual procedure of stripping. However, when eggs or sperm were to be x-irradiated, this was done prior to stripping by x-irradiating the entire fish. From 3 to 6 fish of the same sex were wrapped in cheese cloth, soaked in sea water, and were then placed in a covered plastic fly box measuring 7 cm. in diameter and 2 cm. in depth. The dish was always filled to capacity with fresh sea water during irradiation. Eggs and sperm were stripped from the fish immediately after their exposure.

Early cleavage stages and later embryos were always selected and were x-irradiated in 40 cc. of filtered sea water for each 25 eggs (or embryos) in the same plastic dish, described above. Immediately after irradiation, the exposed material was removed from the sea water and placed in fresh, filtered sea water in order to avoid any possible indirect effect of contamination from extraneous materials irradiated in the sea water. Post-irradiation eggs or embryos were kept 5 per finger bowl in 100 cc. of filtered sea water which was changed every 3–4 days. All material was kept at the laboratory temperature, which was generally about 25° C.

The x-ray facilities used were those available at the Marine Biological Laboratory, Woods Hole, Massachusetts, and consisted of two alternate parallel tubes

[1] This work was aided by a grant from the Office of Naval Research which covered the cost of the research facilities at the Marine Biological Laboratory.

[2] Working under contract AT-30-1-GEN-70 for the Atomic Energy Commission.

run at 182 KVP and 25 MA and having equivalent filtration of 0.2 mm. Cu. The maximum output of this machine at the closest position of the tubes (position "A") gave 6000 r/minute, air dose. The position "B" gave a lower rate of 600 r/minute. For long exposures at the higher rate an electric fan was directed to the container in order to dissipate any heat emanating from the tubes themselves.

Observations and Experimental Data

X-irradiation of the unfertilized egg

The unfertilized eggs of Fundulus were exposed within the body of the fish to various levels of radiation from 1000 r to 200,000 r. They were then stripped into a dry suspension of fresh, normal sperm. These eggs were flooded within 15 minutes with filtered sea water and were studied side-by-side with unirradiated control eggs which had been fertilized simultaneously by normal sperm from the same source.

X-irradiated eggs, whether exposed to 1000 r or to 200,000 r, showed separation of membranes to form a perivitelline space, generally considered as one of the criteria of successful fertilization. It is true that unfertilized eggs placed in sea water will often show this membrane separation after a period of time, but these irradiated eggs showed the effect almost immediately. This occurred even though cleavage did not follow. Eggs exposed to 100,000 r and placed in sea water without benefit of sperm tended to form blastodiscs but there was no real activation. The eggs appeared to be deformed.

Eggs exposed to 100,000 r were often fertilizable with normal sperm and some developed at least as far as stage 15 of Oppenheimer (1937) without any evidence of gross morphological defect (see Figs. 1, 2). These embryos developed optic vesicles, at least 4 pairs of somites, and pericardial vesicles. Some of these embryos reached stage 17 in 46 hours, but all exhibited lordosis and reduced development of the anterior neural structures (see Figs. 3, 7 and 10). No corpuscles appeared in the experimental embryos, while the simultaneous controls had reached stage 22 and had circulating corpuscles. Some such embryos from 100,000 r x-irradiated eggs lived fully two weeks, finally acquiring pulsating hearts but still no corpuscles. They had retinal pigment but were never able to hatch from their surrounding membranes (see Fig. 4). Since the controls (Fig. 5), under identical conditions of temperature, light and sea water, did develop into free-swimming fish fry, we must admit to considerable retardation in development in consequence of x-irradiation of the egg alone. The failure to hatch may have been due to irradiation damage to the hatching enzymes.

Eggs exposed to 50,000 r showed less retardation of development and better morphological differentiation than those exposed to 100,000 r. However, some of these were also without anterior neural differentiation, even though the posterior end and tail development appeared to be quite normal.

X-irradiation of the sperm

X-irradiation of the sperm to very high doses did not have so devastating an effect upon the subsequent development. Sperm exposed to 200,000 r and used to fertilize normal eggs caused development to reach, by 13 days, a stage which compared favorably with the development of the controls at 12 days (compare

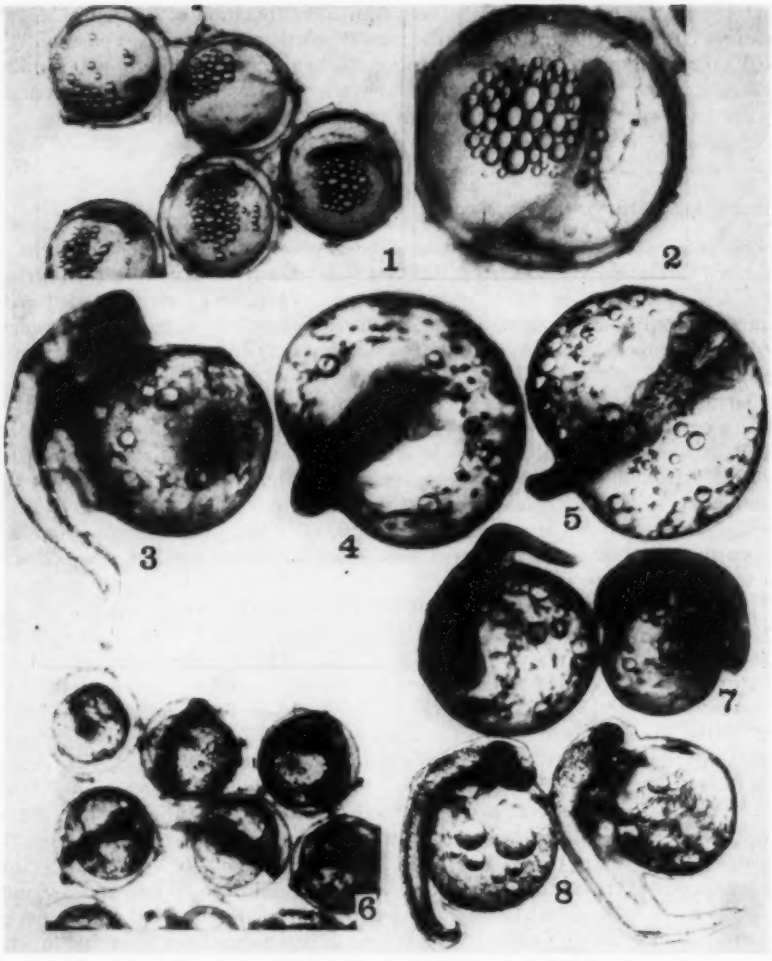

PLATE I. X-Irradiation of sperm and eggs of *Fundulus heteroclitus*.

FIGURE 1. Normal eggs of *Fundulus heteroclitus* fertilized by sperm after exposure to 100,000 r x-rays. Development comparable to stage 15.

FIGURE 2. Single specimen from previous group which developed to stage 17 in 46 hours, while controls developed to stage 22.

FIGURE 3. Development to 11 days of normal egg fertilized by sperm after exposure to 100,000 r. Note reduction in yolk mass, failure of anterior structures, and lordosis.

FIGURE 4. Sperm exposed to 200,000 r prior to use in fertilizing normal eggs of Fundulus. Note better development than in Figure 3, with distinct optic vesicles and chromatophores. Such embryos had pulsating hearts but the circulatory system was devoid of corpuscles. This specimen was 13 days of age, to be compared with control in Figure 5 at 12 days.

Figs. 4 and 5). Retinal pigmentation and pulsating hearts developed, but no blood corpuscles were formed, and none of these embryos ever hatched.

Following 100,000 r x-rays to the sperm, prior to their use in fertilizing normal eggs, some 33% of the eggs cleaved, and many of these developed to stage 12 within 34 hours. Their anterior neural structures developed but were twisted. There were no true somites. Some of these embryos eventually reached stage 30 and hatched, appearing to be quite normal. These may well have been haploids. A few lived for 19 days and all showed cyclopia without other neural differentiation.

X-irradiation of early cleavages and the embryo

The 1- to 2-cell stage is the most vulnerable to x-irradiation insult. Following as little as 300 or 400 r there was stunted but otherwise normal development (Fig. 9). Following 600 r some 25% of the embryos finally hatched, but all were very much stunted. Following 1000 r all were grossly abnormal and without any neural differentiation. The controls had reached stage 22 when the irradiated embryos had reached a stage best identified as stage 15.

The 2- to 8-cell stages were only slightly less vulnerable. The embryos exposed to 500 r exhibited quite normal development.

Stage 11, which is the expanding blastula stage, is slightly more radioresistant than are the early cleavage stages, so that it took 1,000 r to produce a slight quantitative effect on early development (Figs. 10–13). Only a very few of these hatched, however. This may indicate again that the enzymes necessary for hatching are even more sensitive than are some of the morphogenetic processes, for the embryos contained within the chorionic membranes were quite normal. Above 1000 r there appeared all kinds of teratologies, and rarely did development proceed beyond stage 21 (Figs. 14–17). The anterior neural structures were again the ones most seriously affected. However, it must be emphasized that within any group of similarly x-irradiated embryos there was a wide divergence of effect on development.

Point irradiation

By means of a silver point coated with polonium and mounted in a ball-point pen holder, it was possible to spot irradiate with alpha particles a single cell or a limited region of a developing embryo. While irradiation dosage could not be determined accurately, it was estimated that at point of contact the cell would be exposed to about 10,000 reps per minute. A non-radioactive but similar silver

FIGURE 5. Control at 12 days, normal sperm and egg of Fundulus, raised under identical conditions as above experimentals.

FIGURE 6. Eggs exposed to 50,000 r x-rays and then fertilized with normal sperm and allowed to develop for 5 days. Retarded from stage 29 of the controls to about stage 25, with poor development of the anterior structures.

FIGURE 7. Eggs exposed to 100,000 r x-rays and then fertilized by normal sperm and allowed to develop for 4 days. Should be stage 26 but gross abnormalities evident. Compare with Figures 3 and 4.

FIGURE 8. Same as Figure 7 but allowed to develop for 10 days. Should be stage 32, probably comparable to stage 26. Note rather normal development of the eyes, but abnormal head anterior to the eyes and curvature of the tails. Artificially removed from covering membranes.

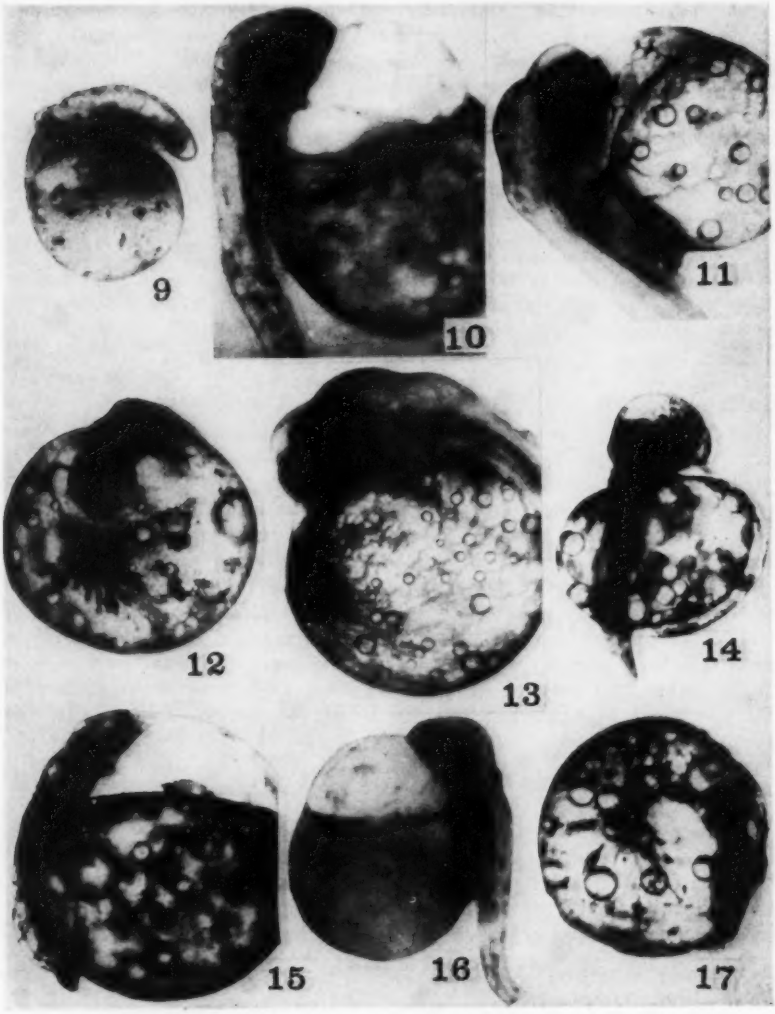

PLATE II. X-irradiation of the early embryo of *Fundulus heteroclitus*.

FIGURE 9. Fertilized egg x-irradiated to 400 r at the one- to two-cell stage and allowed to develop for 10 days. Should be in stage 31 but shows only faint evidence of structural development, poorer anteriorly than posteriorly.

FIGURE 10. Stage 11 from same batch as Figures 11–17 experimentals (expanding blastula at 14 hours) showing normal development at 8 days with head structures straightening out, and well developed and functioning circulatory system (note heart).

FIGURE 11. Stage 11 exposed to 500 r and allowed to develop for 8 days. Development slightly stunted but quite comparable to control in Figure 10.

point was used as control. This method of spot irradiation with polonium was probably first used by Lacassagne and Desclin in 1928.

Eggs were dechorionated [3] and placed in a shallow depression slide. The point of the polonium pencil was brought into direct contact with one of the cells of the two-cell stage and held there for two minutes. This would represent an exposure of approximately 20,000 reps, but the penetration was not more than 30 microns.

The cleavage pattern of the cells was not immediately altered by such spot irradiations. One, or more frequently two, apparently normal cleavages succeeded exposure. This would seem to indicate that there is a latent period of about an hour's duration before the early cleavages can be affected by this dosage of alpha irradiation. Subsequently, however, the progeny of the irradiated cell ceased to divide, although the unirradiated portion continued to cleave in the usual manner. These eggs usually developed into high blastulae which had the appearance of normal blastulae except that they covered less surface of the yolk and the area of contact with the yolk was oval in shape rather than round. By this time the irradiated cells seemed to be no longer a part of the blastoderm but had flattened out and fragmented. At the time of gastrulation no trace of the damaged cells could be found and the experimental eggs were indistinguishable from the controls.

Gastrulation usually began in the unirradiated eggs, and ordinarily no differences were observed until the blastoderm had reached the equator of the egg. After this point, however, there appeared constrictions and lines of tension in the unincorporated yolk of the irradiated eggs. As epiboly continued, the yolk was under such pressure from the tension of the inadequate blastoderm that there appeared bulges of yolk, which often ruptured. Though these ruptures were able to heal over in some cases, only one embryo was observed to complete gastrulation and finally develop into a normal but small fish fry.

When the polonium point was applied to the hindbrain of the dechorionated embryo at stage 25, the embryos failed to develop beyond stage 27, although similarly treated controls, using the dummy pencils, developed normally.

Discussion

As Solberg (1938b) pointed out, the embryos of Fundulus exhibit decreasing radiosensitivity with increasing age. Greatest sensitivity was shown by the fertilized egg and the early cleavage stages, which were retarded substantially by as small a dose as 300 r in our experiments. Later stages acquired increasing radio-

[3] With the much-appreciated technical assistance of Dr. J. P. Trinkaus of Yale University.

Figure 12. Stage 11 exposed to 1000 r x-rays and allowed to develop for 5 days. Note failure of development of blood vascular system.

Figure 13. Same as Figure 12, photographed at 8 days to show functioning blood vascular system but retardation of anterior structural development.

Figure 14. Stage 11 exposed to 1500 r and allowed to develop for 5 days. Note abnormal yolk, and edematous brain vesicle.

Figure 15. Stage 11 exposed to 2000 r x-rays and allowed to develop for 7 days. Extreme stunting but large pericardial vesicle, even though no circulating cells were formed.

Figure 16. Same as Figure 15, photographed the next day. Note rather normal eye development and heart, but no circulating blood cells.

Figure 17. Stage 11 exposed to 2500 r and allowed to develop for 5 days. Extreme stunting and failure of both neutral and circulatory development.

resistance, the blastula developing fairly normally after 1000 r and the 8-day embryo tolerating up to 3000 r without a marked increase in the percentage failing to develop normally.

Contrary to Solberg's results, however, were our observations after irradiation of the unfertilized eggs or the sperm. Using doses of 2000 r to the gametes, Solberg found abnormalities to be produced in 68% of the embryos when the eggs were irradiated and in 86% when only the sperm were treated. He found (1938a) that the testes of Oryzias were 3 or 4 times as radiosensitive as were the ovaries. In our laboratory, we employed much higher doses of irradiation, *i.e.*, 100,000 r and 200,000 r to the gametes of each sex. We found that the relative number of developing embryos resulting from the fertilization of normal eggs by irradiated sperm was greater than the number developing from irradiated eggs and normal sperm. Furthermore, some of the irradiated sperm produced fairly normal embryos, some of which eventually, in the case of those receiving 100,000 r, were able to hatch into viable fish fry. In no case were those embryos developing from eggs irradiated to these high doses able to develop circulating blood corpuscles or hatch.

The disparity of our results from those reported by Solberg may perhaps be explained on the basis of the doses of x-irradiation used. It is still conceivable, upon consideration of our data, that his assumption of greater sensitivity of the sperm is substantiated. Perhaps the x-ray doses which we gave were sufficiently high to destroy the genetic functions of the sperm so that parthenogenetic development followed. Nuclear constituents of cells are more radiosensitive than is the cytoplasm, and the sperm, while retaining their motility, could have been so damaged that the chromosomes were no longer functional. In this case, the irradiated sperm would have activated the eggs, initiating development, yet they would not have contributed genetically to the embryos. Hence the resulting offspring would have been haploid. In Solberg's work, abnormalities probably resulted from the attempt of damaged sperm chromosomes to achieve syngamy.

It is concluded from our observations following the alpha irradiation of cleavage stages in Fundulus that the mechanism of gastrulation was not impaired by the early destruction of one half the germinal material. The process began in the irradiated eggs as it did in the controls. Many investigators (Morgan, 1893, 1895; Lewis, 1912; Hoadley, 1928) have performed experiments in which one cell of the two-cell stage was surgically excised, and the resulting cell was able, if it survived the operation, to form a blastoderm which gastrulated normally and eventually hatched. Morgan presumed that he had not removed as much as half the embryonic material in his experiments. In such a case, any increase in the amount of cytoplasm would probably produce a corresponding increase in the embryo's chances for successfully completing the gastrulation process. It is thought that our failure to achieve normal closure of the blastopore (except in one instance) was due to the great quantitative reduction in blastodermal material. Normally the blastoderm encircles and covers the yolk (Trinkaus, 1951) but here its amount was reduced by half while the amount of yolk remained unchanged. If it were possible to remove some yolk and thereby restore the original ratio of blastoderm to yolk, it is conceivable that more of the irradiated eggs would have been able to complete gastrulation. Attempts to accomplish this were unsuccessful.

Summary and Conclusions

1. X-irradiation of unfertilized eggs of *Fundulus heteroclitus* often caused the separation of the outer membrane to form a perivitelline space, but none of these eggs proceeded to cleave.

2. X-irradiation of males up to 200,000 r did not destroy the motility or the fertilizing power of all the spermatozoa.

3. Eggs exposed to 100,000 r within the body of the female were sometimes fertilizable with normal sperm and many developed as far as stage 17 without gross teratologies. While lordosis was frequent, due to imperfect development of the central nervous system, the pulsating heart was generally found functioning without benefit of corpuscles.

4. Sperm x-irradiated to 200,000 r and used to fertilize normal eggs could often produce quite normal development to 12 days, when the embryos were comparable to the controls at 11 days. These may have been haploids, due to a genetically non-functional nucleus.

5. An exposure of the spermatozoa to 100,000 r frequently resulted in quite normal embryos that were able to hatch.

6. The first (or earliest) cleavage is the most vulnerable to x-irradiation insult, such embryos tolerating a maximum of 300 r without gross abnormalities. Following 1000 r the embryos were unable to achieve any neural differentiation.

7. A dose of 1000 r to stage 11 (expanding blastula) was qualitatively comparable to a dose of about 500 r to the first cleavage stage.

8. The anterior neural structures appear to suffer most following x-irradiation of any embryonic stage from the first cleavage through the expanding blastula. Pulsating hearts without corpuscles are often found.

9. Point x-irradiation of a single cell of the two-cell stage with polonium (20,000 reps) appears to damage the cell to such an extent that regulation and successful gastrulation cannot be achieved, except in rare cases. This may be due to mechanical factors, or to a quantitative alteration of the blastoderm-yolk relations.

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ON THE DISTRIBUTION OF NEREIS DIVERSICOLOR IN RELATION TO SALINITY IN THE VICINITY OF TVÄRMINNE, FINLAND, AND THE ISEFJORD, DENMARK

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In the literature on the salinity tolerance and osmotic behavior of the widespread estuarine polychaete, *Nereis diversicolor* O. F. Müller, there occur suggestions such as that of Ellis (1937) that physiologically distinct races exist in different regions. Ellis had noted that *N. diversicolor* from Roscoff, France, differed from worms of the same species from Plymouth and Bangor in Britain in rate of weight regulation in response to suddenly lowered salinity, and suggested that the physiological differences observed were of "racial rather than environmental" origin. Unfortunately, Ellis did not report the conditions of salinity under which his experimental animals had existed in nature nor even the salinities at the time and place of collection, so that one cannot decide whether the observed differences in response might reasonably be considered of adaptive significance. Similarly, certain of the findings of Beadle (1937) of differing responses in winter and summer cannot be evaluated because the pattern of salinity variation in the Blythe estuary from which he obtained his worms is not stated.

It is obvious that *N. diversicolor* as a species experiences, and is presumably adapted to withstand, markedly different salinity conditions in different parts of its geographical range. In Britain the species is most characteristic of estuaries, in which it may experience extreme tidal and seasonal variations in salinity, whereas in the tideless Baltic Sea it encounters over much of the year a low but relatively stable salinity. Since the larvae of *N. diversicolor* tend to be benthic (Dales, 1950), populations in British estuaries must be fairly well separated from each other and from those on the continental coasts, and have certainly been isolated for a long time from these of the inner Baltic. Because conditions of extreme salinity variation must present different selective pressures than do conditions of stable low salinity, *N. diversicolor* seems a favorable species in which to investigate the possible existence of physiological races, differing from each other in their tolerance of low salinities. In other words, the character of the distribution of this species is such as to favor the formation of populations genetically distinct in respect to physiological response to low salinity. But while thus suggesting that physiological races in *N. diversicolor* may be a reasonable possibility, we should bear in mind that sound evidence for such races is almost non-existent. In this discussion a physiological race is thought of as a population in which a characteristic and adaptive level of physiological performance exists in response to a given environmental factor, in this instance low salinity. It seems clear that before one can claim to have demonstrated a physiological race of this sort, one

must have demonstrated a clear difference in response to the variable factor under comparable conditions in the separate localities, and must also know the pattern of variation of this factor in the different portions of the range of the species. The problem thus divides itself naturally into laboratory studies to measure the response to the controlled variable, in this case low salinity, and field studies to determine the salinity conditions under which *N. diversicolor* lives in representative parts of its geographical range.

The writer has had the opportunity to carry out such studies upon the ecology of *N. diversicolor* and upon its chloride regulation at several selected localities in northwestern Europe, localities which represent three extremes of the salinity conditions within the range of the species.

1. A "marine-dominated" habitat has been studied at Millport, Scotland, and the results reported in a previous paper (Smith, 1955). In brief, the above study (which has been supported by observations in the Salcombe estuary in southern England) shows that *N. diversicolor* when in an essentially "marine" environment finds its optimum in local, relatively brackish, zones.

2. "Stable low salinity" represents perhaps the opposite extreme of the ecological range of *N. diversicolor*. Such conditions are best exemplified in the mesohaline[1] waters of the Baltic Sea, with which the present paper is largely concerned.

[1] Owing to the number of salinity classifications which have been proposed, it is necessary to state that in this paper reference is to the system of Redeke as amended by Välikangas (1933), expressed as salinity in parts per mille (‰). More recently Ekman (1953) has proposed a more complex terminology which embodies essentially the same divisions as the previous one, as seen in the following comparison:

Välikangas (1933)		Ekman (1953)	
Fresh water	< 0.5 ‰	Fresh water	< 0.5 ‰
Oligohaline	0.5–3.0	Oligohaline brackish	0.5–3.0
β (meio-) mesohaline	3.0–8.0	Mesohaline brackish	3.0–10.0
α (pleio-) mesohaline	8.0–16.5	Polyhaline brackish	10.0–17 (20?)
Polyhaline	16.5–30.0	Oligohaline sea water	17 (20?)–30
Ultrahaline (sea water)	> 30.0	Mesohaline sea water	30–34 (?)
		Polyhaline sea water	> 34 (?)

I have elected not to follow the Ekman system because it seems more likely than the older system to lead to confusion as a result of repetition of adjectives, by the use of generally accepted terms in new quantitative meanings, and by the restriction of the term "brackish" to a portion of the range of salinities which it has been taken to cover during long usage. There is much to be said for retaining the general terms oligo-, meso-, and polyhaline to describe waters of low, intermediate, and higher salinities, respectively, and keeping "seawater" for these salinities above 30‰ which characterize the open seas (here some modification of the Välikangas system may be needed). "Brackish" can be omitted as a classificatory term, and the boundaries of the oligo-, meso-, and polyhaline left somewhat flexible, to be adjusted by zoogeographers to fit local conditions (in which it is necessary to state specific salinities anyway). It seems possible that the upper limit of a mesohaline body of water, as judged on faunistic grounds, would be somewhat different in a warm brackish sea than it is in the Baltic, to which both of the above systems rather specifically apply.

It should be noted that the limits of the meio- ("less") and the pleio- ("more") mesohaline of Välikangas have been determined on the basis of Baltic faunas; these divisions thus represent

3. Another type of extreme is represented by "estuarine" habitats such as are common in Britain. Here salinity *variation* is the predominating characteristic, the salinity changing semi-diurnally with tides, often semi-monthly with the lunar cycle, seasonally or erratically with winds and rainfall, and topographically according to relative volumes of estuary bed and fresh-water inflow. Conditions of salinity in the estuarine habitat of *N. diversicolor* are difficult to characterize in any simple way (Bassindale, 1943a, gives one excellent account), and in connection with the present work will be discussed in a later paper.

The results of the comparative physiological work which has been carried out concurrently with observations upon the distribution of *N. diversicolor* in the several areas visited will be assembled in a single paper elsewhere, but it seems advisable to summarize the ecological studies on each type of habitat separately. The attempt to carry out both field and laboratory studies in several widely separated localities within the space of a year has necessarily resulted in a superficial picture. Nevertheless, these studies are probably the first attempt to see *N. diversicolor* with the same eyes over so wide an ecological range, and to support laboratory studies of its salinity tolerance by systematic field observations. It is hoped that our picture of this species may be somewhat clarified, if only by defining gaps in our knowledge. The present paper reports observations made on the south coast of Finland, not far from the eastern limit of *N. diversicolor* in the Baltic Sea, together with subsequent observations in a Danish fjord of comparably stable but not so low salinity. A summary of ecological conditions in each area has been included to complete the picture and as an aid to future visiting workers.

Part I. Observations at Tvärminne, Finland

General description of the area

The Baltic Sea is not only the largest and best-known brackish-water area of the world, but offers in its practically tideless condition a most illuminating contrast to estuarine waters with their tidal fluctuations in level and salinity. For the present study the Tvärminne Zoological Station of Helsinki University has proved most favorably situated. Located on the southeastern side of the peninsula of Hangö at the southern tip of Finland (Fig. 1), it lies near the eastern limit of the range of *N. diversicolor* (Välikangas, 1933), in the west-east gradient of decreasing salinity of the Gulf of Finland. This Gulf is essentially a continuation of the central Baltic, not being cut off (as is the Gulf of Bothnia) by a shallow sill or group of islands. The region about Tvärminne has been extensively studied, so that much information is available to support the studies of the short-term visitor. Thus the bottom fauna of the area has been studied by Segerstråle (1933a, 1933b, 1949, 1955), the larger aquatic vegetation by Luther (1951a, 1951b), the bottom micro-fauna by Purasjoki (1945), the hydrography and plankton by Halme (1944) and Halme and Kaartotie (1946), as well as by others, references to whom may be found in the papers cited. One of the regular water-sampling stations of The Institute of Marine Research, Helsinki, lies in Tvärminne Stor-

a regional adaptation of the more widely applicable but less precise term "mesohaline." Ekman's divisions seem to embody the same ideas, but have gone beyond the concept of a characteristic (or "true") brackish-water fauna to express the idea that the waters inhabited by this characteristic fauna are the "true" brackish waters, a somewhat doubtful logical step.

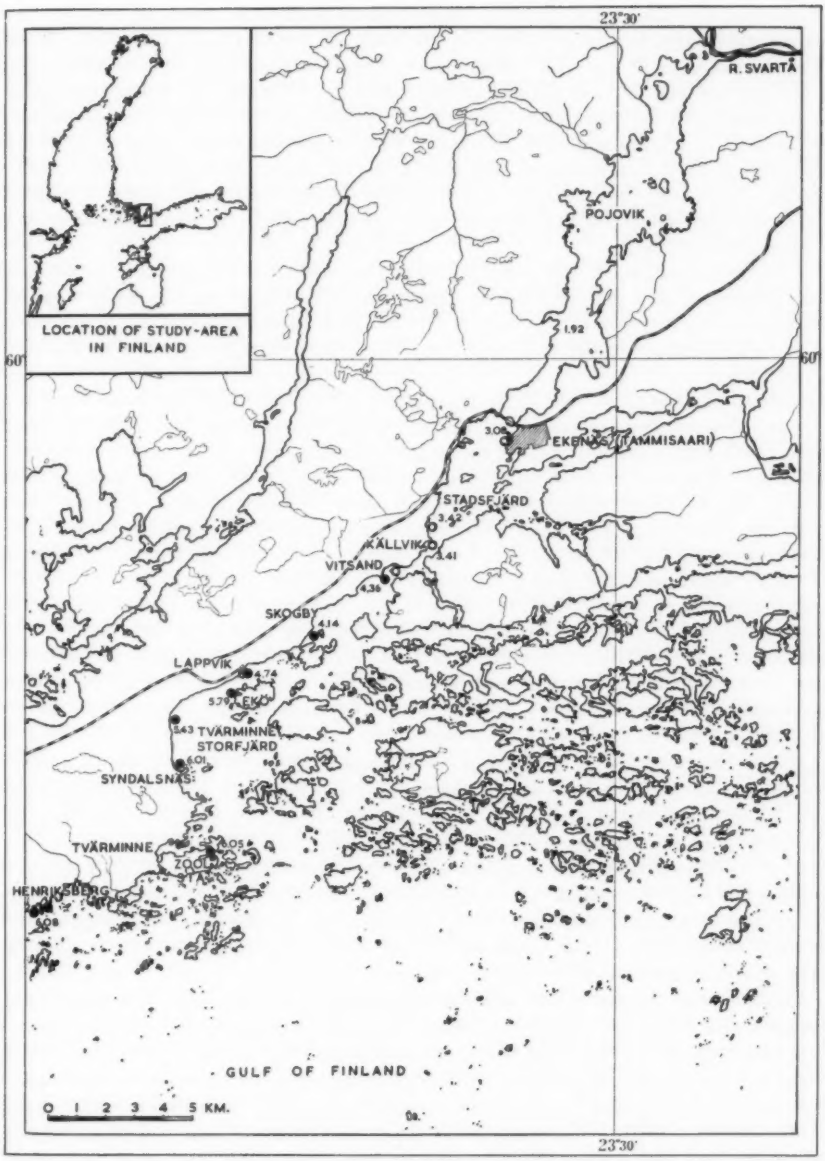

FIGURE 1. Map of the Tvärminne area, Finland, showing localities mentioned in text. Numerals indicate surface salinities observed in July–August, 1954. Black circles, *N. diversicolor* present; open circles *N. diversicolor* not found after careful search on suitable bottom. Detailed data in Table I.

fjärd near the Zoological Station; hence nearly complete series of salinity and temperature records are available for the past 25 years (*e.g.*, Granqvist, 1951). Summer salinities of the open coastal waters near Tvärminne have been about 6‰ at the surface and 7‰ at 30 meters (*loc. cit.*). In the past 15 years the region, in common with the rest of the Baltic, has experienced a noticeable rise in salinity of 0.4–0.7‰ (Granqvist, 1949, 1952). This is not known to have affected the distribution of *N. diversicolor*, but the breeding ranges of certain animals, *e.g.*, *Aurelia*, have been extended eastward (Segerstråle, 1951a), while the amphipod *Calliopius laeviusculus* has extended its range shoreward in the Tvärminne area (Segerstråle, 1953).

Salinity variations in the coastal water mass are quite marked at certain seasons and have been summarized by Segerstråle (1951b). Mainly as a result of the large fresh-water discharge of the River Neva, salinities along the south coast of Finland decrease to the east, and are subjected to further lowering at the time of melting snow. In addition to the main west-east coastwise gradient, there is a gradient of decreasing salinity as one passes northward (inshore) from the outer islands, this gradient being most marked where rivers from the Finnish mainland reach the Gulf. Pronounced salinity stratification may occur, especially when the ice cover prevents mixing by winds. Thus in Tvärminne Storfjärd the salinity of the deeper waters may be almost unaffected by a layer of fresh water from the River Svartå (Fig. 1) flowing beneath the ice (Granqvist, 1938). Its effect upon the bottom fauna of deeper waters must be negligible. Segerstråle (1951b) has shown that spring lowerings of salinity to less than 50% of the annual mean (taken at a depth of 5 meters to avoid the surface layer) occur only about once in 20 years in Tvärminne Storfjärd; the coastal waters are thus of relatively stable salinity. However, in shallow inshore bays the spring dilution may be a much more frequent and serious matter. Thus the Krogarvik, a little bay adjacent to the Zoological Station, whose depth is mostly under three meters and which receives fresh water drainage from a nearby pond and marsh as well as being subjected to the regional lowerings of salinity, may experience salinity reductions to less than 40% of the mean in three years out of four. Segerstråle (1933a) records salinities at the bottom of the Krogarvik as low as 2.02‰ in 1929 and 0.21‰ in 1932, although from 1945 to 1950 none lower than 3.77‰ were recorded (Segerstråle, 1951b). These critical periods of greatly lowered salinity occur in the spring (February–May) and may last as long as a month. The topography of Tvärminne Storfjärd and the Krogarvik are illustrated in Segerstråle's papers (1933a, Abb. 1; 1949, Fig. 4), where more complete descriptions of the region are available.

Tidal changes in water level are virtually absent. A tide of 4 cm. is statistically demonstrable at Hangö (Lisitzin, 1945), but variations caused by local winds and by general water levels in the Baltic are much greater. Thus, during the period of the present study (July 5 to August 9, 1954), the water level at the Zoological Station varied over a range of 40 cm. and averaged 10.3 cm. above mean water level. Luther (1951a) discusses water levels and related matters in some detail, and points out the serious effects which may be caused by low water coincident with the spring discharge of fresh water beneath the ice at shallow inshore localities.

Although to the zoogeographer the distribution of *N. diversicolor* in the west-

east salinity gradient of the south coast of Finland is of primary interest, the physiological ecologist with limited time at his disposal finds the local gradient of decreasing salinity from the open Baltic inshore towards the land more convenient for study. The region about Tvärminne receives no fresh water streams of any consequence except for the River Svartå, which discharges a large flow into the northern end of the long fjord-like Pojovik (Fig. 1). This fresh to oligohaline water passes out over deep-lying mesohaline water in the depths of the Pojovik, where the salinity at 30 meters approximates 4‰ (Halme and Kaartotie, 1946), and over a shallow (7 m.) sill at Ekenäs (Tammisaari) into the Stadsfjärd. The latter bay shows a distinct salinity stratification in summer. Halme (1944) describes a labile β-mesohaline wedge of deeper water entering the Stadsfjärd via the narrow but relatively deep (15 m.) strait at Källvik, sometimes extending inwards past the Ekenäs sill and at other times being displaced seaward by the massive outflow of oligohaline water. The Källvik strait thus represents a topographical interruption or discontinuity in the salinity gradient between Ekenäs and Tvärminne Storfjärd, a point to be amplified later.

Segerstråle (1933b) reported that *N. diversicolor* occurred regularly but sparsely on sandy bottoms down to depths of 6–8 meters and occasionally on soft bottoms. The maximum density (44 per m.^{2}) and weight (7.4 gm. per m.^{2}) were obtained on a muddy bottom. Although reported from a number of stations, the species does not appear from this report to have been as abundant at that time as it is at present. Since Segerstråle's stations were restricted to the area about Tvärminne and the southern part of the Storfjärd, the limits of the salinity range of *N. diversicolor* were not known, and a main objective of my field work was to establish the distribution of the species in relation to the local gradient of decreasing salinity inshore from Tvärminne.

Field observations

In the course of collecting worms for physiological work, and on a limited number of other days, water samples were taken at points indicated on Figure 1, and a careful search made for *N. diversicolor*. Quantitative sampling could not be used in the time available; rather, as careful a search as possible was made at each spot on the most suitable bottom, and a rough estimate of abundance based upon the time and effort used in securing specimens. Negative results, where indicated, are based upon at least two visits to the site on separate days under good collecting conditions, employing both visual search and digging in shallow water and dredging in deeper water. All collecting stations were at sites where the bottom appeared to be, on general grounds, suitable for *Nereis*. The most favorable substratum was clearly sandy, ranging in consistency from decidedly coarse on exposed beaches to fine, gray, and firm on protected shores. At deeper levels, worms could be found in mud or gyttja (a soft deposit rich in organic matter), usually with some admixture of sand. Contrary to Segerstråle's experience (1933b) I have obtained the largest specimens in firm sand rather than in mud and at depths of less than one meter. Worms were not taken on clay bottoms, such as were encountered along much of the shoreline of the Stadsfjärd.

Samples were preferably dug with a spade in water up to waist-deep, and washed through a sieve of about one-mm. mesh. On open sandy bottoms it was

TABLE I

Summary of field observations in Tvärminne region

Scale of exposure
E —Exposed
Pr—Protected
SP—Semi-protected

Scale of abundance of Nereis
R—Rare; occasional or single individuals
S —Scattered; consistently found by careful search; 5–10 worms per hour of digging or hand dredging
C—Common; 1 or 2 worms per spadeful or dredge haul
P—Plentiful; several worms per spadeful or dredge haul

Scale of size
L —Large
M —Medium
Sm—Small

Collecting site (see Fig. 1)	Surface salinity ‰	Exposure and substratum	*Nereis diversicolor*		Associated flora (conspicuous elements only)	Associated fauna (conspicuous elements only)
			Abundance	Size		
Henriksberg (outside point)	6.08 (5.95–6.21)	E; coarse sand	S	L	Flocculent or encrusting algae on sand; *Fucus vesiculosus* on rocks	*Macoma baltica* (abundant) *Mya arenaria* *Mysis vulgaris*, etc.
Henriksberg (inside point)		SP; medium sand	F	M and Sm		
The Krogarvik (Zool. Sta.) head and north shore	6.05 (5.99–6.10)	Pr; shallow; fine firm gray sand	S	L	*Fucus vesiculosus* (allochthonous) *Chorda filum* (autochthonous) *Potamogeton perfoliatus* *Ranunculus obtusiflorus* *Myriophyllum spicatum* *Chara tomentosa* *Phragmites communis*	*Cardium edule* (large), *Macoma* (all sizes) *Mytilus edulis* (small), *Mya* (small) *Lymnaea ovata*, *Theodoxus fluviatilis* *Corophium volutator*, *Pontoporeia affinis* *Mesidotea entomon*, *Gammarus* sp. Chironomid larvae, *Dendrocoelum*, etc.
Central parts		Pr; depth 2–3 meters; mud, gyttja, plant remains	C	M and Sm		
Syndalsnäs	6.01	SP; medium sand	P	M	*Phragmites* (otherwise as at Henriksberg	As at Henriksberg
Shore south of Lappvik	5.43	E; coarse sand	C	M	As at Henriksberg but more sparse	*Mya* (large), *Macoma* (small and scarce) *Bathyporeia pilosa*, etc.
Behind point at Lappvik	4.74	SP; medium sand, slightly muddy	C	M and L	Sparse algal mat *Potamogeton*, *Phragmites*, etc.	As at Henriksberg

TABLE I—*Continued*

Collecting site (see Fig. 1)	Surface salinity ‰	Exposure and substratum	*Nereis diversicolor*		Associated flora (conspicuous elements only)	Associated fauna (conspicuous elements only)
			Abundance	Size		
Small bay on Ekö	5.79	Pr; muddy sand below *Fucus*	S	M	Autochthonous *Fucus*, other algae *Phragmites*	*Macoma*, *Mesidotea*, *Lymnaea stagnalis*, etc.
Below Skogby	4.14 (4.09–4.18)	SP; fine muddy gray sand	S	M	Some autochthonous *Fucus* *Phragmites*, etc.	*Macoma* (large and abundant) *Mya* (medium), etc.
Vitsand	4.36 (4.00–4.72) (5 m) 4.81 (10 m) 6.08	SP; fine sand and clay in shallows; mud, coarse sand and debris at 3–5 meters	R; 1 at 4–5 meters	M	*Phragmites* Allochthonous *Fucus*	*Macoma*, and others as in Krogarvik, but restricted to depth below 3 meters
Källvik passage	3.41 (3.10–3.56)	SP; as at Vitsand	Absent		*Phragmites* Allochthonous *Fucus*	In channel, below 5 meters: *Mytilus* (large and abundant), *Mya* and *Cardium* (medium) *Membranipora crustulenta* and *Balanus improvisus* (large and abundant, on *Fucus* and *Mytilus*) Rest of "marine" fauna as in Krogarvik
Southern end of Stadsfjärd	3.42 (2.97–3.87) (5 m) 5.72	SP; as at Vitsand	Absent		*Phragmites*, *Potamogeton*, and other freshwater plants in shallows Allochthonous *Fucus* below 3 meters	In shallows: *Bithynia tentaculata*, *Theodoxus* *Asellus aquaticus*, chironomid larvae, etc. Below 3 meters: *Balanus* and *Membranipora* (common) *Cardium* (small, 1.2 cm.; free in coarse sand) *Macoma* (all sizes, abundant) *Pontoporeia affinis* (abundant), *Mesidotea* (large and breeding) (*Mya* and *Mytilus* absent)
Ekenäs (Tammisaari)	3.08 (2.66–3.49)	SP; fine gray sand, slightly muddy	Absent (no dredging at this station)		*Enteromorpha* Filamentous green algae	*Cordylophora lacustris* (on pilings); *Macoma* at deeper levels (*Mesidotea* beyond this point at deeper levels as breeding resident of Pojovik)

often possible to locate worms by observing the burrow openings; digging at such spots commonly yielded worms where random digging and sifting had failed to reveal a single individual in an hour's time. Hence calm weather permitting a clear view of the bottom was considered a requisite of good collecting conditions on sandy shores fairly free of macroscopic algae, but was of less value in protected bays where algal growth often covered the bottom. Dredging was carried out from a skiff or motorboat, employing a light triangular ring-dredge whose line was weighted with chain to encourage the dredge to bite into the substratum. This dredge would secure a good sample of soft mud and/or coarse sand but proved useless on the type of fine, hard, sandy bottom (as at the head of the Krogarvik) where spading showed the presence of scattered to common very large worms, rather deeply buried.

Salinity determinations have been made as in the previous study (Smith, 1955) by determining chloride in grams per liter on duplicate one-ml. samples. Because all previous reports on the Tvärminne area have used salinity rather than chlorinity, my results in the present work have been expressed as approximate salinity in parts per mille, calculated by multiplying my chloride (essentially chlorosity) values by 1.81. The results are thus not exactly what would have been obtained by routine hydrographic methods, but are close enough for an ecological account.

The field observations are summarized in Table I and the distribution of *N. diversicolor* and of mean surface salinities (deep salinities in table) shown in Figure 1. It may be seen that in all suitable localities from Henriksberg on the outer shore to the vicinity of Lappvik, worms are fairly common in shallow water, but disappear from the shore somewhere above Skogby. At Vitsand *N. diversicolor* could not be found in shallow water despite the presence of apparently suitable bottom, but a single individual was taken by repeated dredging at a depth of 4–5 meters. The species thus reaches its limit outside the constricted Källvik strait, at a point which, incidentally, is also the limit of autochthonous (attached) *Fucus vesiculosus* (Luther, 1951a). No other reduction in the marine fauna has been noted in the deeper water at this point, and it is significant that at a depth of 10 meters the salinity is still as high as at the surface on the outer shore. In the narrow Källvik strait a vigorous marine fauna, except for *Nereis*, also exists. Within the Stadsfjärd we see a drastic reduction of this fauna, with the dropping out of *Mya* and *Mytilus* and the reduction of *Cardium*, as well as the appearance of a number of fresh water forms.

Discussion

A study of Table I reveals several facts, some of which when viewed in the light of other findings are not a little puzzling:

1. In typical tidal estuaries, marine animals generally tend to occupy an upper intertidal level as they extend upstream into less saline waters. While this may be attributable to differences in the position of suitable substratum (as pointed out by Spooner and Moore, 1940, for mud-dwellers in the River Tamar), it has the advantage that the higher salinities of the flood tide are received and the lower salinities of the ebb avoided. But here in the tideless Baltic, the distribution of the worms (which are of course always "subtidal") follows the zone of favorable salinity to a lower level as fresher waters are approached. The salinities actually

encountered by these worms in summer are thus not so low as the surface salinities over the range might imply.

2. It would appear that *N. diversicolor* in the Tvärminne area does not normally have to endure summer salinities below about 4‰. When we consider that worms of this species living in estuaries have a range extending from salinities of 22‰ or more down to salinities below 1‰ (Percival, 1929), and when we further consider the fact observed in the experimental part of this study that worms from any part of the Tvärminne area can at summer temperatures tolerate soft fresh water (chloride about 0.02‰), it is surprising indeed that *N. diversicolor* is limited in the field at a salinity as high as 4‰. The obvious conclusion is that the species is not being limited by the salinities prevailing in summer near Tvärminne, but if salinity is a limiting factor at all, it must be operating at some other time of year, most probably during the spring reduction of salinity associated with melting snow. Two possible ways in which the species might be affected are (1) by inability of the adults to perform the necessary osmotic regulation when temperatures are near 0° C. or (2) by a failure in some aspect of reproduction and/or dispersal. The first alternative would imply that *N. diversicolor* could not survive the winter or spring even if it had become established in the Stadsfjärd in the previous summer. We have no specific information on which to base further discussion of this possibility. The second alternative implies that *N. diversicolor*, even if capable of surviving in the Stadsfjärd as an individual, would fail to reproduce itself there, and that larvae produced elsewhere are unable to enter to replenish the population. The available information on the breeding habits of *N. diversicolor* (summarized by Dales, 1950) indicates that in most localities it is a winter or early spring spawner. Although the breeding season at Tvärminne is not yet known, it would appear to have been completed prior to July in 1954. In examining and taking coelomic fluid samples from over 100 worms in the laboratory during the present work, besides handling a much greater number in the field, not a single male has been identified. Female worms, greenish and with obviously over-ripe eggs, were found on several occasions, and worms with small undeveloped oöcytes were numerous, probably representing the next year's breeding stock. Small worms (under 15 mm. in length) were common in collections, and many more undoubtedly escaped through the sieves used. The smallest nereid seen (collected by Dr. T. G. Karling at Henriksberg on August 4) measured 2 mm. in length and had 15 setigerous somites. According to the rates of growth reported by Dales (1950) at unspecified temperatures, this worm was probably 6–8 weeks old. Spawning must then have been essentially completed before the summer months, but whether it fell in or after the spring period of low salinity could only have been determined by field studies at that time.

3. Although in British estuaries *N. diversicolor* penetrates further into waters of low salinity than any other "marine" types except for certain crustaceans which do not enter the present discussion (Percival, 1929; "Plymouth Marine Fauna," 1931; Spooner and Moore, 1940; Bassindale, 1943b), this species in the Tvärminne area is one of the first elements of the characteristic brackish-water faunal assemblage to drop out. To be sure, certain marine types such as the amphipods *Pontoporeia femorata*, *Calliopius laeviusculus*, and *Gammarus locusta*, as well as the isopod *Idothea baltica*, the priapulid *Halicryptus*, the polychaete *Harmothoë sarsi*, and others, seem to be restricted to the highest salinities found about Tvär-

minne. These have been poorly or not at all represented in my collections, and will not be included in the following discussion. Leaving them aside, we see from Table I that *N. diversicolor* does not penetrate as far into lower salinities as do the rest of the animals commonly associated with it. To an observer whose experience has been gained outside the Baltic this fact seems paradoxical, but it would appear that the situation in the shoreward gradient of decreasing salinity from Tvärminne to Ekenäs reflects a condition characteristic of the Baltic as a whole and not unusual in terms of this brackish sea. Thus the ranges shown by Välikangas (1933) indicate that in the gradients of decreasing salinity of both the Gulf of Finland and Gulf of Bothnia, *N. diversicolor* stops short of such forms as *Mytilus, Macoma, Membranipora, Corophium volutator,* etc. Other information indicates that *Mya* extends further into the Gulf of Bothnia than does *Nereis* (Segerstråle in litt.), and *Cardium* further east in the Gulf of Finland (Segerstråle, 1933b). One is thus encouraged to hope that an analysis of the factors limiting *N. diversicolor* in the local salinity gradient shoreward from Tvärminne may shed light upon the more general problem of its distribution in the Baltic Sea. And the closer we can come to understanding why the relative distribution of *Nereis* and its associates in the Baltic is so different from that seen in typical estuaries, the closer are we to understanding the effects of these different environments upon the animals which are able to inhabit them, and the nature of physiological adaptation to such environments.

4. With reference to Table I, the following facts and comments are offered: It may be significant that of the associates of *N. diversicolor* which do penetrate the Stadsfjärd, at least two, *Mesidotea entomon* and *Pontoporeia affinis,* are glacial relicts which are also found in fresh water lakes (Segerstråle, 1955). *Membranipora crustulenta* likewise extends into very low salinities in the heads of the Gulfs of Finland and Bothnia (Välikangas, 1933) and so would not be encountering severe conditions in the Stadsfjärd. Of the properly marine species, *Macoma baltica* is a summer breeder (Segerstråle, 1951b) and would be reproducing when salinity in the Stadsfjärd is maximal. A further point to notice is that *Cardium, Balanus improvisus,* and *Membranipora,* even if unable to reproduce within the Stadsfjärd (a point which has not been confirmed or denied), might have entered it as planktonic larvae at a time of saltwater ingression following the spring outflow; Levander (1915) has recorded larvae of the latter two forms within the Stadsfjärd in summer (at his station VIII). It is also possible that these three species could have been carried in as post-larval stages on drifting *Fucus. Membranipora* in the Stadsfjärd is generally on this plant; *Balanus* is commonly so found. One large individual of *B. improvisus* has been found on a clean pebble weighing 27 grams, suggesting settlement *in situ,* but the possibility that the stone was carried in by *Fucus* at some previous time cannot be entirely ruled out. Likewise, very small *Cardium* are abundant, byssally attached to *Fucus* outside the Stadsfjärd, and could readily drop off after drifting in. But in the case of *N. diversicolor,* the possible planktonic stage is at most a brief one (Dales, 1950), and attachment to allochthonous *Fucus* does not seem a probable means of transport. Although Thorson (1946) reports larvae presumed to be of *N. diversicolor* in the plankton of the Danish Øresund from late March to July–August (with maximum abundance in April and May), there are no records of nereid larvae in

the plankton about Tvärminne. In response to a question on this point addressed to Drs. Purasjoki and Segerstråle, the latter has replied (in litt.) "We are of the opinion that there is rather convincing evidence of the worm not having a planktonic stage in our waters. No such larva has been found in the numberless plankton samples taken since decades, including the cold season." We may further note that a nereid larva entirely lacks a protective shell, and may for this reason be less resistant than larvae of *Balanus* or bivalve molluscs. If conditions in and above the Källvik strait are such as to prevent the reproduction of *N. diversicolor* or the survival of its early larvae, and if the species with its brief and benthic larval life lacks the means of colonizing this region beyond its actual breeding range, then we may consider that the distribution of the species is limited not by one, but by the interaction of several factors. More specifically, the factors which limit the spread of *N. diversicolor* (while permitting the advance of its marine associates) are not restricted to the physiological tolerances of the worm and its larvae, but must include physical factors of hydrographic conditions which may not be favorable to dispersal at the time larvae are produced. The data of Halme and Kaartotie (1946) suggest that the narrow Källvik passage is subjected to greater spring disturbances of salinity at its deeper levels than is any other part of the area studied. If we consider the 5-meter depth as a level likely to be critical for *N. diversicolor* (which has not been reported deeper than 6–8 meters), the records of Halme and Kaartotie show that in 1936–37 the Tvärminne Storfjärd had an annual salinity variation of 1.25°/$_{00}$ (6.45–5.20), the Pojovik varied by 1.35°/$_{00}$ (2.30–0.95), while in the Källvik passage the variation recorded was 2.30°/$_{00}$ (5.25–2.95). This indicates a deep penetration of oligohaline water during the spring discharge, and suggests that the constricted Källvik strait represents a rather abrupt discontinuity in the local inshore salinity gradient. It is thus a hydrographic barrier which may affect the distribution of *N. diversicolor* more adversely than it does that of the associated marine species. If, as seems likely, *N. diversicolor* produces its larvae during the spring lowering of salinity, when there is still a large net outflow past Källvik, there is a good chance that even if the weakly-swimming larvae were injected into the plankton by turbulence, they would either be killed by low salinity or swept seaward. Other marine species with a later breeding season or a longer planktonic existence might be able to pass into the Stadsfjärd when the hydrographic barrier is minimal during the summer. It would be interesting to know if a more gradual salinity gradient leading into some other Baltic river system might not permit *N. diversicolor* to extend its range into salinities as low as it seems to tolerate in estuaries elsewhere. If such a river can be found, it might provide a test of the ideas just expressed.

Conditions in the Krogarvik, the shallow bay close by the Zoological Station, might also prove very illuminating in respect to the possibility that *N. diversicolor* is either limited by inability to regulate osmotically during periods of minimal salinity combined with low temperature in spring, or that its reproduction or larval survival are hindered. Unfortunately, the determinations of salinity at the bottom of the Krogarvik obtained by Segerstråle in the years 1929–32 and 1945–50 have not been continued until 1954. *N. diversicolor* was common in this bay in the summer of 1954, and the largest individuals, which had certainly over-wintered, were found near the head of the bay in water so shallow that they must have been

close beneath the ice (unless they had migrated from deeper levels, an undemonstrated possibility). It is perhaps significant that the winter of 1953–54 was unusually free of snow, and it may be that the Krogarvik experienced little reduction of salinity at the spring thaw. I am informed by Professor Palmgren that the taking of salinity samples at the bottom of the Krogarvik will be resumed, and if the winter results can be correlated with the abundance of *N. diversicolor* in each following summer, it may be possible to determine from this natural experiment what salinities can be tolerated by these worms at winter temperatures. Studies of osmoregulatory ability under refrigeration in summer might be helpful, but probably not conclusive because of the ability of some animals to adjust their metabolism to seasonal changes in temperature; studies made in winter would be more informative.

FIGURE 2. Map of the Isefjord, Denmark.

Part II. Observations in the Isefjord, Denmark

General description of the area

In a geographical sense the Isefjord (Fig. 2), in the northern part of the island of Zealand to the west of Copenhagen, is intermediate between British waters and the Baltic, while its mean surface salinity of 20‰ is midway between salinities at Tvärminne and Plymouth. The hydrography of the Isefjord has been summarized by Nielsen (1951). His account shows that this body of water is characterized by a stability of salinity greater than that of the south Finnish coast. The Isefjord has an area of 280 square kilometers and is so shallow (mostly less

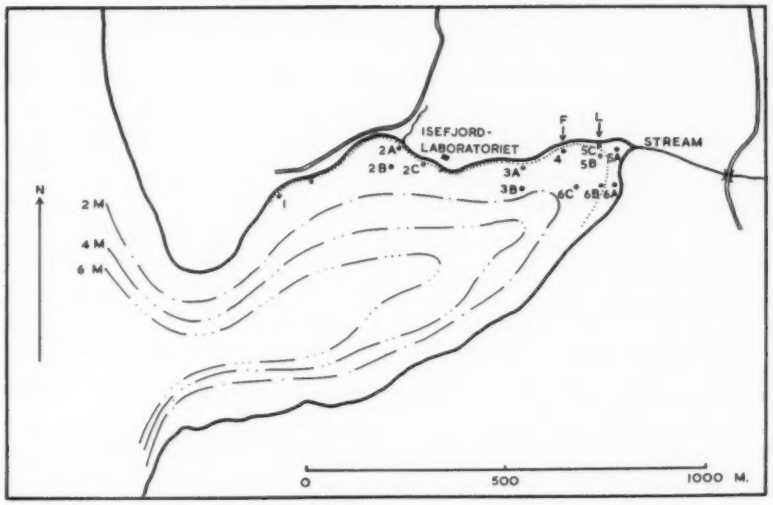

Figure 3. Vellerup Vig, showing sampling stations. Details in Table II. Arrows marked F and L show limits of *Fucus* and *Littorina* on the shore.

than 10 meters) that despite the presence of a sill at its mouth, salinity stratification rarely occurs. According to Nielsen's observations, mean surface salinity in the Inner Broad (southern part of the fjord) is 20.18‰, with extremes noted of 17.79 and 22.23‰, while at depths of 4–6 meters the corresponding values are 20.54‰ (19.03–23.88). The Isefjord is thus polyhaline in the sense of Välikangas (1933). In comparison with conditions near Tvärminne it is notable that the lowest salinity of 17.79‰ was obtained in March, immediately beneath the ice, and that the difference in salinity between surface and 5 meters at that time was only 4.74‰. The Isefjord freezes easily and may have an ice cover for three months in cold winters, although there is evidently not such a marked seasonal lowering of salinity beneath the ice as is experienced in Finnish waters. The reason for this is doubtless the slight fresh water drainage into the Isefjord, which has a very small watershed (800 sq. km.) and receives only small streams. So low is the fresh water input that the Inner Broad, the head of the fjord, actually has a mean salinity 0.5‰ *higher* than the Outer Broad which lies nearer the

TABLE II

Field observations, Vellerup Vig: salinities at low and high water at sampling stations (see Fig. 3) densities of Nereis diversicolor and N. southerni (approx. number per ¼ sq. meter), and principal associated biota

Key to symbols

F—*Fucus vesiculosus* (autochthonus)
L—*Littorina littorea* (on upper midtidal rocks)
Mt—*Mytilus edulis*
M —*Mya arenaria*
C —*Cardium edule*

Station (see Fig. 3)	Character of station	Salinities °/₀₀		Worm density		Associates
		L. W.	H. W.	*N. div.*	*N. south.*	
1	At L. W. M.; muddy sand	18.40	—	6	1	F L Mt — —
2A	Subtidal, by stream mouth; sand and clay	1.63	17.58	83	4	F L Mt — —
2B	50 meters out; muddy sand, near rocks	15.41	18.40	2	33	F L Mt C —
2C	Subtidal, away from stream; sand and clay	—	—	3	22	F L Mt — —
3A	Subtidal; muddy sand	10.96	13.67	3	41	F L Mt — —
3B	50 meters out; fine sand	12.33	17.21	0	19	— — — M C
4	Subtidal, at limit of *Fucus;* sand	7.73	9.59	75	2	F L Mt — —
5A	Intertidal, under flowing fresh water at low tide; gravelly	0.03	0.62	6	0	— — — — —
5B	Subtidal, at foot of fresh water outflow; gravelly	0.29	0.83	39	0	— — — — —
5C	Intertidal; muddy sand, at limit of *Littorina* on rocks	1.25	4.13	76	0	— L — — —
6A	Intertidal; muddy sand at head of bay	2.32	9.37	134	0	— — — — —
6B	Subtidal; muddy sand	1.88	—	47	1	— — — M —
6C	100 meters from H. W. M.; fine sand	4.05	9.52	5	16	— — — M C

Kattegat. Tides are small, about 20 cm. in mean range, but massive ingression or expulsion of water during gales commonly causes deviations in water level of ± one meter. The Isefjord is thus an area of moderately reduced but exceptionally stable salinity, and it is of interest to learn where *N. diversicolor* finds its optimum within it.

Field observations

The present study has been restricted to the head and northern shore of Vellerup Vig, a small bay on the eastern shore of the Inner Broad (Fig. 3). The

intertidal shore is mostly narrow, rather steeply sloped, varying from muddy sand to coarse gravel, covered with stones in more exposed places, and interrupted by extensive patches of reeds (*Phragmites*). A soft flat muddy shore about 25 meters wide is exposed by low tides at the head of the bay. Below low-water mark the bottom is nearly flat, of fine muddy sand with some clay and scattered large boulders which support *Fucus vesiculosus*, *Mytilus edulis*, and *Littorina littorea*, except in the less saline head of the bay. *N. diversicolor* occurs quite generally along the midtidal shore beneath stones resting on muddy sand, but is not abundant sub-tidally except in certain areas which are clearly those freshened by inflowing streams. A good-sized stream enters the bay at its head and a small one just to the west of the Isefjord Laboratory.

In making collections, most worms had to be dug under water, hence direct sampling of quadrats was not feasible, and the method was adopted of filling a cubical box 25 cm. in each dimension by several spadefuls of substratum taken

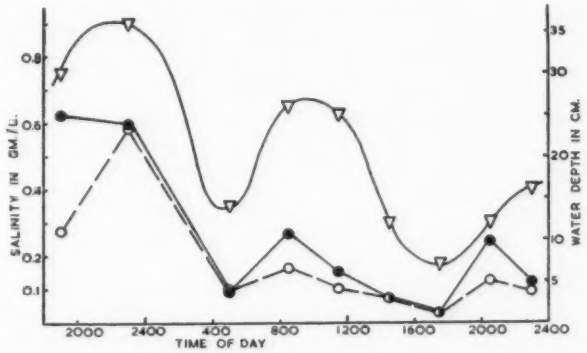

FIGURE 4. Variation in water level and salinity over fringe of population of *N. diversicolor* at station 5A (see Fig. 3) at stream mouth, Vellerup Vig, August 28–29, 1954. Triangles, depth of water in cm.; black circles, salinity at bottom; open circles, salinity at surface.

within an area a few feet across at each station. On the assumption that the digging was effective to about half the depth of the box, the full box would represent an area sampled of ⅛ square meter. The material obtained was sifted through a pair of sieves of ¼- and ⅛-inch mesh. Most of the smallest worms were lost, but the counts obtained are comparable among themselves. Figure 3 shows the positions of collecting stations, and the results of the field observations are summarized in Table II. It is evident that *N. diversicolor* is highly concentrated in the head of the bay and near the mouth of the small stream near the laboratory.

A second nereid, which has been shown to be less tolerant of reduced salinity (Jørgensen and Dales, in press), is also abundant in Vellerup Vig and is being described as a new species, *Nereis southerni*, by Abdel-Moez and Humphries (in press). The populations of *N. southerni* and *N. diversicolor* overlap only very slightly. I am informed by Dr. Erik Rasmussen that *N. southerni* has been increasing in abundance in Vellerup Vig during the past few years, and that it

seems to have replaced *N. diversicolor* in areas where the latter used to be of regular occurrence. This fact forces one to exercise caution in seeking to establish the salinity optimum of *N. diversicolor*. The apparent optimum shown in the present studies can only be a restriction of the species to that part of its potential range where it escapes critical interspecific competition. The same problem has been encountered in the case of *N. diversicolor* at Millport, Scotland (Smith, 1955). It is, nevertheless, clear that *N. diversicolor* at Vellerup exhibits a marked tolerance for water of very low salinity, even extending its distribution into areas exposed to outflowing fresh-water at low tide.

At the mouth of the stream at the head of the bay *N. diversicolor* was found in reduced numbers, but still commonly, living intertidally beneath flowing fresh-water for part of each tidal cycle. In order to gain a rough picture of the salinities endured by this vigorous fringe of the population at the extreme of its habitat, water-levels and salinities were measured at intervals over a 27-hour period, and the results are shown in Figure 4. Although no great regularity is revealed, it is evident that these worms can live beneath salinities of less than 1‰ (0.65–0.03‰) for at least a day at a time. It should be noted that the summer of 1954 prior to these observations had been the rainiest experienced in many decades, so that salinities at this spot had probably been even lower than those recorded. On the other hand, it is likely that an occasional high stand of water caused by wind may have provided a temporary respite in the form of a saline wedge forced in beneath the outflowing fresh-water at this point. Figure 4 indicates some salinity stratification near the stream mouth.

Discussion

N. diversicolor collected from the head of Vellerup Vig has been used by the writer in studies of chloride regulation and also by Jørgensen and Dales (in press). Worms from this spot are quite capable of living in fresh water in the laboratory, and apparently some of them endure it for a greater or less time in nature. In my studies of the level of chloride regulation after adaptation to low salinities, I have found no difference between *N. diversicolor* from Finland and from Denmark. This finding of similar regulatory ability is in marked contrast to the distribution of the species in the respective localities. In the Finnish area of prevailing low salinity we find *N. diversicolor* restricted to the more saline part of the potentially available range, even less able to invade oligohaline waters than *Mytilus, Cardium, Mya, Balanus improvisus*, and others. But in the Isefjord, where prevailing salinities are three times higher, *N. diversicolor* is strongly concentrated in the least saline portions of the available range, into which it advances further than any of its marine associates, thus behaving quite like its relatives in British estuaries and unlike those of the inner Baltic Sea.

The factors which determine optimal conditions for a species are complex, and the distribution of *N. diversicolor* in relation to salinity has not, by this study, been reduced to any simpler terms. We have seen that the relative position of the species in the regional salinity gradient is quite different in the Baltic Sea than it is elsewhere, but it is quite possible that the differences in distribution are caused by special hydrographic conditions (in particular, the spring reduction of salinity characteristic of the inner Baltic) rather than by any fundamental or

"racial" differences between the worms themselves. The gradient between the polyhaline waters of the Isefjord and the oligohaline conditions near stream mouths is interrupted by no such zone of hydrographic instabliity as the Källvik Strait imposes between the mesohaline and oligohaline waters of the Tvärminne region. It is possibly quite significant that the most unfavorable hydrographic conditions both in the Tvärminne area and in the Baltic as a whole occur at or near the probable time of reproduction of *N. diversicolor*. We have also noted that although the Isefjord as a whole has a mean salinity of about 20‰, which lies within the optimum salinity range of *N. diversicolor* as seen at Millport (11–22‰), the Vellerup population finds its apparent optimum at a much lower absolute salinity. But in both instances the apparent salinity optima may be in part the result of interspecific competition. The studies at Tvärminne and Vellerup, despite their limited scope, emphasize the fact that an ecological optimum must be related to the total life history and physiological capabilities of the species concerned.

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Summary

1. A comparative field study of the distribution of *Nereis diversicolor* near Tvärminne, south Finland and in the Isefjord, Denmark shows that although the species penetrates into nearly fresh water in the Isefjord, its distribution near Tvärminne is limited at summer salinities of over 4‰.

2. The apparent salinity optimum of *N. diversicolor* on the border of the polyhaline Isefjord is lower than on the "marine-dominated" beach at Millport, Scotland, but in both instances there may be a restriction of the potential range because of interspecific competition.

3. The paradoxical situation is seen near Tvärminne of *N. diversicolor* being among the first of the characteristic brackish-water fauna to drop out as salinity

lessens, whereas in the Isefjord as well as in British estuarine situations it penetrates further toward fresh water than the rest of its associates.

4. It is suggested, on the basis of hydrographic conditions in the Tvärminne area, that salinities prevailing in summer cannot be the limiting factor for *N. diversicolor* in the Baltic Sea, but that critically low salinities occurring in spring while temperatures are still very low may adversely affect the osmoregulation and/or reproduction of the species. Furthermore, there may be a hydrographic (as well as a purely physiological) barrier to the spread of the species into oligohaline waters, in which connection the time of breeding and the lack of a long planktonic stage in the life history may be important.

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STUDIES ON SHELL FORMATION. IV. THE RESPIRATORY METABOLISM OF THE OYSTER MANTLE[1, 2]

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One of the major problems in the study of shell formation is the relation of respiratory pathways to the deposition of the $CaCO_3$ and the organic matrix. In addition to the participation of respiration in the synthesis of organic constituents, respiration may also be involved in the transport of organic and inorganic substances through the cells of the mantle tissue to the site of shell deposition. Further, CO_2 from respiratory intermediates may provide a source of shell carbonate. Since the respiratory pathways of the shell-forming tissues of mollusks are unknown, the first requirement, and one of the objectives of this series of studies, is their identification. The present study concerns oxidative phases, and includes assays of oxidative enzymes and the effects of various substrates and respiratory inhibitors on oxygen consumption. Decarboxylation reactions will be the subject of another report.

It has long been recognized that the mantle is intimately concerned in processes of shell formation. Recently Hirata (1953) demonstrated that the isolated mantle of the oyster has the capacity to deposit both the organic and inorganic portions of the shell, and that this capacity is maintained for a relatively long period after isolation. The fact that the mantle has within itself the mechanisms of shell formation means that *initial* studies of the respiratory reactions can be confined to this structure. Once the respiratory activities of the mantle are established, one will then be in a position to relate these to the deposition of shell substances.

Methods

The earlier portions of the respiratory studies were carried out on oysters from East Bay, Pensacola, Florida, during the period February to May, 1952, and continued on oysters from Core Creek, Beaufort, N. C., during July to November, 1952. Specimens of *Crassostrea virginica* (formerly *Ostrea virginica*), 8.0 cm. to 11.5 cm. in length (average 9.6 cm.), were collected in deep water and maintained

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below low tide level at the laboratory. Temperature and salinity ranges were as follows: Beaufort: temperature, 17.2°–31.0°; salinity 24.6–35.2; Pensacola: temperature, 10.0°–27.5°; salinity, 18.8–27.0. Enzyme assays were performed on Beaufort oysters from Core Creek and North River during May to July, 1953. Since the salinity near the laboratory where the oysters were maintained was higher than at the collecting sites, all oysters were held from 10 to 14 days before being used. During this period the temperature range was 20°–30° and the salinity range was 27.6–34.1.

Respiration measurements

Immediately after taking the specimens from the water the mantles were removed; cut into strips; drained for 10 seconds with one end touching filter paper; and weighed on a torsion balance. Respiratory measurements were carried out using Warburg's direct method on single strips of mantle weighing 150–220 mg. in 5-ml. flasks with sea water as the medium. The total fluid volume including tissue was 1.1 ml. The mantle is sufficiently thin that diffusion of oxygen is not limiting. Flasks were gassed with O_2 bubbled through sea water. All measurements were made at 25.0° except where noted. The pH of the medium was routinely measured prior to and frequently following runs. In the majority of cases the pH was not altered appreciably during the course of the respiratory measurements even with shaking for periods of 5–7 hours duration in the presence of alkali. Occasionally, however, there was a marked increase in the alkalinity from an initial pH of about 8 to 9.1–9.5. In a few cases a change of several tenths of a pH unit in the acid direction was also observed.

All substrates and inhibitors were made up in sea water. Adjustment of pH was made with HCl and NaOH. In studying the effect of a compound on respiration, O_2 consumption was measured during a 2- to 3-hour control period prior to the addition of the experimental compound from the side arm. After addition, measurements were continued for 3 to 4 hours. Oxygen consumption remained constant throughout the seven-hour period of these measurements but decreased slightly during the next few hours. The procedure recommended by Robbie (1948) was followed in using NaCN.

Enzyme assay methods

Aconitase. Spectrophotometric method of Racker (1950) using supernates from fresh homogenates. Homogenates were prepared by grinding a pooled sample of mantle tissue in a mortar followed by homogenization in 0.1 *M* K phosphate buffer, pH 7.4, in a glass homogenizer. The tissue was kept ice cold throughout. The supernatant following 10 minutes of light centrifugation was used for the enzyme assays. The increase in optical density at 240 mμ resulting from the conversion of citrate to unsaturated cis-aconitate was followed at intervals of 30 seconds. A unit of enzyme activity is the amount causing an increase in optical density of 0.001 log unit per minute at 22°–26°. Tissue activity is expressed as units of enzyme per mg. dry weight.

Isocitric dehydrogenase. Spectrophotometric method of Mehler *et al.* (1948) using an acetone powder extract as prepared by Wenner *et al.* (1952), with slight

modifications, in glycylglycine buffer, pH 7.2. The reaction is started by the addition of isocitrate, and the increase in optical density at 340 mμ resulting from the reduction of TPN is read at 15-second intervals against a control cuvette containing all components except isocitrate. Enzyme activity is expressed in units per mg. of acetone powder, one unit being the amount of enzyme required to increase the optical density 0.01 log unit per minute at 22°–26°.

Succinic dehydrogenase. Spectrophotometric method of Cooperstein, Lazarow and Kurfess (1950). Assays were carried out on homogenates prepared with 10 volumes of 0.03 *M* Na phosphate buffer, pH 7.4, followed by further dilution, giving a final concentration of 1 : 30 in the cuvette. The increase in optical density at 550 mμ resulting from the reduction of cytochrome *c* was read at 30-second intervals. Measurements were made immediately following preparation of the homogenate and before clumping of particles became marked. Enzyme activity is expressed as the decrease in the logarithm of the molar concentration of oxidized cytochrome *c* per minute for a 1 : 150 tissue dilution.

Cytochrome oxidase. Spectrophotometric method of Cooperstein and Lazarow (1951). The tissue was prepared as for succinic dehydrogenase except that the final dilution was 1 : 300. The decrease in optical density at 550 mμ resulting from oxidation of cytochrome *c* was read at 30-second intervals. Enzyme activity was calculated as the decrease in the logarithm of the molar concentration of reduced cytochrome *c* per minute for a 1 : 100 tissue dilution.

Fumarase. Spectrophotometric method of Racker (1950) using a fresh homogenate and also an acetone powder extract as prepared by Wenner *et al.* (1952). The increase in optical density at 240 mμ resulting from the formation of the unsaturated fumarate from malate at pH 7.4 provides an index of enzyme activity. The calculation of unit enzyme activity is the same as for aconitase.

Malic dehydrogenase. Spectrophotometric method of Mehler *et al.* (1948) using an acetone powder extract. The reaction was started by the addition of oxaloacetate, and the decrease in optical density at 340 mμ resulting from the oxidation of $DPNH_2$ was read at 15-second intervals. A pH of 7.2 was maintained with glycylglycine buffer. The calculation of enzyme activity is the same as for isocitric dehydrogenase.

Oxaloacetic decarboxylase. The manometric method of Vennesland *et al.* (1947) was employed using fresh homogenates at pH 4.5. Corrections were made for spontaneous breakdown of the substrate. Activity is expressed as μl. CO_2 per mg. dry wt. per hr. as calculated from CO_2.

Results

Endogenous respiration

Before considering the individual enzymes of the mantle and the effect of respiratory substrates, attention will first be given to endogenous respiration including the respiration of the different portions of the mantle. The possibility of metabolic differences between mantle areas is suggested by structural differences, differences in calcium turnover rates (Jodrey, 1953), and by differences in the rate of calcium deposition between areas of the inner shell surface (Wilbur and Jodrey, 1952). A difference in CO_2 content of mantle areas of the clam *Venus mercenaria* has also been reported (Dugal, 1939).

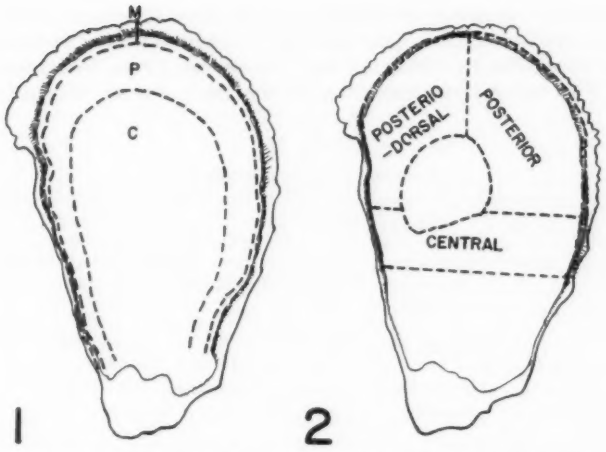

FIGURE 1. Mantle marked off to show zones used for respiratory measurements on Florida oysters. M, marginal zone; P, pallial zone; C, central zone.

FIGURE 2. Mantle marked off to show regions used for respiratory measurements.

For experimental purposes the oyster mantle may be considered to consist of three concentric zones (Fig. 1): a thin outer folded margin (M); an area attached to the shell and here termed the pallial zone (P); and a larger, thinner central zone (C). Measurements of endogenous respiration were carried out on the three zones (Table 1). The pallial zone exhibited the highest rate and the central the lowest. Differences between zones were significant at the 1% level. In subsequent experiments mantle regions (Fig. 2) including all three zones were utilized for respiratory measurements. The endogenous respiration in North Carolina oysters was the same for the three mantle regions. In Florida oysters the three regions appeared to exhibit differences in respiration, but the data, which were obtained under conditions of temperature and season quite different from the North Carolina oysters, are not sufficiently extensive to warrant discussion. In order that any such difference would not figure in the results, experimental and control tissues were always taken from corresponding mantle regions.

TABLE I

Endogenous respiration of mantle zones

Mantle zone	μl. O_2/mg. wet wt./hr.
Marginal	0.13±0.03
Pallial	0.15±0.03
Central	0.12±0.02

Mantles were divided into three zones as shown in Figure 1 and the oxygen consumption of the parts measured simultaneously. Florida oysters. Environmental temperatures, 10°–14° C. Measurements were carried out at constant temperatures between 18° and 21°. Salinity 24–25 parts per thousand. Figures show means and standard deviations, $\sqrt{\frac{\Sigma d^2}{n-1}}$.

A further aspect of endogenous respiration deserves mention, namely, the effect of short-term starvation of isolated mantles. Right mantles with the attached shell were isolated (Hirata, 1953) and kept for periods of one to 7 days in running sea water. The endogenous respiration of the three regions shown in Figure 2

TABLE II

Respiratory enzymes in mantle

Enzyme	Mantle	Mammalian tissue		Reaction
Aconitase	none	mouse liver rat kidney rat liver	33 .50 25*	$\text{citrate} \xleftrightarrow{\pm H_2O} \text{aconitate} \xleftrightarrow{\pm H_2O} \text{isocitrate}$
Isocitric dehydrogenase	0.36	mouse liver rat kidney rat liver	10.8 66 22*	$\text{isocitrate} + \text{TPN} \xleftrightarrow{+2H} \text{oxalosuccinate} + \text{TPNH}_2$
Succinic dehydrogenase	0.03	rat liver	2.96	$\text{succinate} + \text{ferricytochrome c} \xleftrightarrow{\pm 2H} \text{fumarate} + \text{ferrocytochrome c}$
Cytochrome oxidase	0.61	rat liver rat kidney	11.85 23.16	$\text{ferrocytochrome c} + 1/2\ O_2 \longrightarrow \text{ferricytochrome c} + H_2O$
Fumarase	2.0± 0.3	mouse liver rat kidney rat liver	132 62 77*	$\text{fumarate} \xleftrightarrow{\pm H_2O} \text{malate}$
Malic dehydrogenase	3.3	mouse liver rat kidney rat liver	256 173 87*	$\text{malate} + \text{DPN} \xleftrightarrow{+2H} \text{oxaloacetate} + \text{DPNH}_2$
Oxaloacetic decarboxylase	592± 187	mouse liver	3.2	$\text{oxaloacetate} \xleftrightarrow{\pm CO_2} \text{pyruvate}$

Aconitase: supernatant from light centrifugation of 1:20 homogenate in 0.1 *M* K phosphate buffer, pH 7.4, 0.1 ml.; 0.05 *M* phosphate buffer, pH 7.4, 2.4 ml.; 0.03 *M* sodium citrate, 0.5 ml. *Isocitric dehydrogenase:* 0.25 *M* glycylglycine buffer, pH 7.2, 1.0 ml.; 6.6×10^{-4} *M* TPN, 0.45 ml.; 0.05 *M* $MnCl_2$, 0.05 ml.; 1:15 acetone powder extract, 1.0 ml.; 0.01 *M* sodium isocitrate, 0.5 ml. *Succinic dehydrogenase:* 0.1 *M* sodium succinate in 0.1 *M* Na phosphate buffer, pH 7.4, 0.5 ml.; 0.03 *M* NaCN in 0.17 *M* phosphate buffer, pH 7.4, 0.2 ml.; 1:10 homogenate in 0.03 *M* phosphate buffer, pH 7.4, 1.0 ml.; 100 mg.% cytochrome in 0.17 *M* phosphate buffer, pH 7.4, 1.0 ml.; water to 3.0 ml. *Cytochrome oxidase:* 60 mg.% cytochrome in 0.03 *M* phosphate buffer, pH 7.4, 3.0 ml.; 1:10 homogenate in 0.03 *M* phosphate buffer, pH 7.4, 0.1 ml. *Fumarase:* 0.5 ml. of 1:40 homogenate in double distilled water; 0.1 *M* potassium malate, 1.5 ml.; 0.1 *M* K phosphate buffer, pH 7.4, 0.75 ml.; double distilled water, 0.25 ml. *Malic dehydrogenase:* 0.25 *M* glycylglycine buffer, pH 7.2, 1.0 ml.; 1.7×10^{-3} *M* $DPNH_2$, 0.2 ml.; 1:15 acetone powder extract, 0.1 ml.; 0.01 *M* sodium oxaloacetate 1.0 ml., water to 3.0 ml. *Oxaloacetic decarboxylase:* 0.1 *M* acetate buffer, pH 4.7, 0.25 ml.; 6.6×10^{-4} *M* TPN, 0.05 ml.; 10^{-2} *M* $MnCl_2$, 0.1 ml.; 1:10 homogenate in 0.1 *M* acetate buffer, 0.5 ml.; 7.6×10^{-2} *M* oxaloacetate, 0.1 ml. (1 mg. per flask).

Figures for mantle enzymes other than fumarase and oxaloacetate represent pooled samples of 9 or more mantles. Starred figures on mammalian tissues obtained in the present study. Other data on mammalian tissues from Wenner *et al.* (1952) except succinic dehydrogenase and cytochrome oxidase which are from Cooperstein *et al.* (1950) and Cooperstein and Lazarow (1951), respectively.

was then measured. Surprisingly, the respiration per mg. wet weight did not change significantly with the period of isolation, nor was it different from that of mantles taken directly from unstarved oysters. Such metabolic stability, especially in the absence of added substrate, is indeed remarkable.

Enzyme assays

Table II presents the results of the assays of mantle enzymes, together with values for the same enzymes in certain mouse and rat tissues for comparison. Isocitric, succinic, and malic dehydrogenases were present and confirm earlier unpublished experiments on succinic and malic dehydrogenases carried out in collaboration with Dr. Henry Kritzler and Mr. Arthur A. Hirata using the Thunberg technic. Fumarase was found in fresh homogenates but not in acetone preparations. Assays of freshly prepared homogenates gave no indication of aconitase activity.

Close comparisons between the *in vivo* activity of the mantle and mammalian enzymes (Table II) are not justified even though the same assay methods were used for both. Under the conditions of assay, however, tricarboxylic cycle enzymes in mantle have a considerably lower activity than in the mammalian tissues.

TABLE III

Effect of substrates on mantle respiration

Substrate	0.005 *M*	0.01 *M*	0.025 *M*	0.05 *M*
Isocitrate	——	26±8	——	——
Citrate	−1±5	2±4	——	——
Succinate	10±5	15±9	18±8	26±3
Malate	13±5	20±5	16±7	——
Oxaloacetate	13±8	——	20±7	22±5
Pyruvate	8±10	3±7	——	——

Figures show mean percentage change in oxygen consumption and standard deviation following the addition of the substrates to mantle.

On the other hand, this is not the case for oxaloacetic decarboxylase. This enzyme had an activity in the mantle which is two orders of magnitude higher than that of mouse liver. This finding may be of special significance for the mantle tissue which is concerned with processes of carbonate deposition. (See paper by Wilbur and Jodrey which follows the present paper.)

Substrate effects

Several substrates of the tricarboxylic acid cycle have been added to pieces of mantle (Fig. 2) and their effects on oxygen consumption measured (Table III). Isocitrate, succinate, malate, and oxaloacetate stimulated respiration, the effect becoming greater as the concentration was increased above 0.005 *M*. The maximal stimulation amounted to 20–26%. Oxidative enzymes for these four substrates were demonstrated in the previous section (Table II). Citrate was without effect, and this result is correlated with the failure to demonstrate aconitase. In the absence of aconitase citrate would not be converted to isocitrate which can

be oxidized by the mantle. Pyruvate also did not alter oxygen consumption appreciably. The possible "sparking" action of acetate was not studied.

The question of penetration of the substrate arises in cases of the negative results with pyruvate and especially with citrate which would form calcium and magnesium salts in the sea water medium.[4] Ronkin (1950) found that the effect of respiratory inhibitors on the gill of the mussel *Mytilus* was increased by decreasing the pH of the medium, presumably from increased penetration of the undissociated molecule. The effect of pH was examined with succinate, pyruvate, and citrate (Table IV). Pyruvate and citrate exhibited no significant effect on the mean respiration over the range pH 6.0–8.4. However, certain mantles were stimulated and others were inhibited as indicated by the standard deviation values. The stimulating action of succinate remained constant between pH 6.6 and 8.4 and was abolished below pH 6.6, although the level of endogenous respiration was

TABLE IV

Effect of pH on mantle respiration

Substrate	pH				
	6.0–6.2	6.3–6.5	6.6–7.4	7.5–7.9	8.0–8.4
Succinate, 0.05 *M*	2.1 ±11 (6)	0.1 ±6.8 (4)	24 ±11 (6)	26 ±7.5 (12)	27 ±9.2 (7)
Pyruvate, 0.01 *M*	−6.2 ±5.7 (2)	——	−2.2 ±11 (2)	——	2.5 ±10 (2)
Citrate, 0.01 *M*	2.1 ±6.1 (11)	——	1.5 ±12 (5)	——	1.6 ±20 (5)
Sea water	0.15	0.20	0.21	0.18	0.17

Oxygen consumption was first measured for 2–3 hours in sea water, the pH of which had been altered by the addition of HCl or NaOH. Substrates made up in sea water of the same pH were then tipped in and the measurements of oxygen consumption were continued for several hours. The pH was again measured at the end of the experiment. The mean percentage change and standard deviation of the respiratory rate resulting from the addition of substrate are given. O_2 consumption in μl/mg. wet wt./hr. Figures in parenthesis show the number of cases. At pH 6 the calculated dissociation is 74% for succinate and 80% for citrate. Data were not available for a calculation for pyruvate.

maintained very nearly at the normal level to a pH of 6.0–6.2. This latter result poses an interesting problem in that one might expect endogenous respiration to be depressed to a greater degree if succinate is in the respiratory cycle.

We have mentioned that isolated mantles can be maintained in good condition for several days without added substrate. Nine such mantles isolated for 2 to 7 days have been compared with 7 freshly dissected mantles with respect to their respiration in the presence of 0.05 *M* succinate. The mean respiration (μl O_2/mg. wet wt./hr.) was 25 and 23, respectively ($P < 50$), demonstrating that both groups respond similarly. This result is not especially surprising in view of the unchanged endogenous respiration.

Respiratory inhibitors

In the two previous sections the presence of succinic dehydrogenase and cytochrome oxidase in mantle has been demonstrated. One can therefore expect that compounds known to inhibit the succinoxidase system will affect mantle respira-

[4] However, isocitrate, which would be expected to behave similarly, apparently penetrates.

tion. The action of such enzyme inhibitors and the copper-complexing agent diethyldithiocarbamate has been examined and the results are presented in the following sections.

Sodium cyanide. Endogenous O_2 consumption of mantle in the presence of 2×10^{-4} *M* NaCN averaged only 7% of the control value without cyanide. The inhibitory action of cyanide was completely reversible after an exposure of 1¼ hours (Fig. 3) but only partially reversible after longer periods.

Methylene blue, which typically reverses at least a portion of cyanide inhibition (Thunberg, 1918), increased the oxygen consumption in a saturated solution in sea water from the cyanide level of 7% to a new level of 10%, and thus was

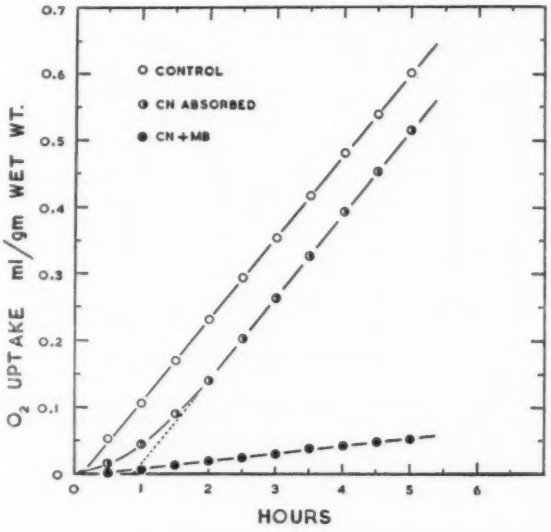

FIGURE 3. The effect of cyanide on mantle respiration. Lower curve: mantle in 2×10^{-4} NaCN; saturated solution of methylene blue (final concentration 1.4×10^{-5} *M*) tipped in at one hr. The change from 7% of control respiration to 10% after the addition of methylene blue is not perceptible on the graph. NaCN present in center well to prevent absorption of cyanide by KOH. Middle curve: mantle in 2×10^{-4} *M* NaCN for 1¼ hr. in Warburg flask prior to 0 time on graph. At 0 time KOH without cyanide was added to center well of flask to absorb HCN. Oxygen uptake returned to the control value as cyanide concentration decreased.

without appreciable effect. This result is not due to failure of penetration since uptake of methylene blue by the cells of the whole mantle can be clearly seen under the microscope. Methylene blue when added to normal mantles neither altered the endogenous respiration nor the increased oxygen consumption produced by succinate. The absence of an increased succinate effect in the presence of a saturated solution of methylene blue (6.3×10^{-5} *M*) was shown in 12 experiments in which the average respiratory increase amounted to 21.2% as compared with 20.5% for 0.005 *M* succinate alone. From this result it appears that the cytochrome system is not limiting in succinate oxidation.

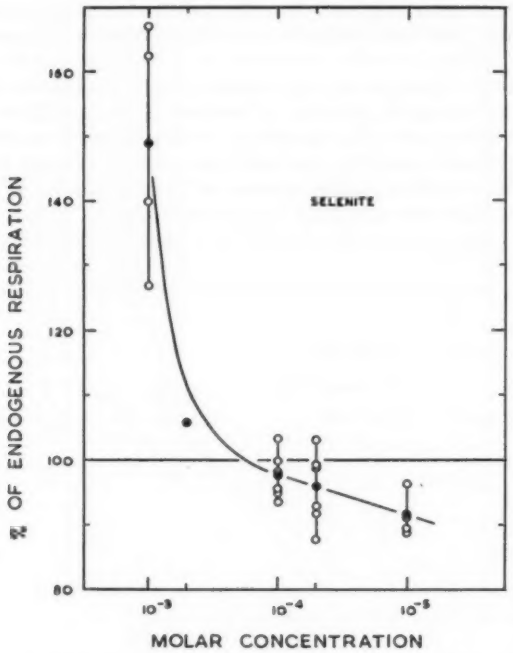

FIGURE 4. The effect of sodium selenite on mantle respiration. Solid circles show mean values. Open circles give values for individual experiments. Endogenous O_2 uptake was measured for 1–2 hrs.; selenite was then tipped in and measurements were continued. Note that the abscissa is in log units.

TABLE V

Effects of diethyldithiocarbamate and ethyl carbamate on mantle respiration

	Mean per cent inhibition		
	10^{-4} *M*	10^{-3} *M*	10^{-2} *M*
Diethyldithiocarbamate	8	32*	13
	16	27	14
	10	22	7
	21	26	0
Ethyl carbamate	4	3	5
	−1	1.5	4

The figures represent individual experiments and show the percentage change in O_2 consumption. North Carolina oysters.

* Maximum inhibition of 57% was found in Florida oysters.

Sodium selenite. Selenite has been studied in view of its inhibition of succinic dehydrogenase (Stotz and Hastings, 1937). The results of selenite on endogenous respiration are summarized in Figure 4 which demonstrates a dual effect of this compound. A slight inhibition occurred with 10^{-5} *M* with progressively less inhibition as the concentration was increased; and at 10^{-3} *M* a mean increase in respiration of 49% was observed. Measurements using selenite alone in sea water showed that the autoxidation of this compound would not account for the observed increase in oxygen consumption.

Selenite in a concentration of 10^{-5} *M*, which depressed endogenous respiration slightly, had a variable effect on respiratory increase from added succinate. In some cases the selenite reduced or abolished the succinate effect; in others it did not.

Sodium malonate. Malonate characteristically inhibits succinic dehydrogenase competitively. Mantle tissue treated with 0.025 *M* malonate prior to the addition of 0.005 succinate showed a 38% inhibition of the succinate effect which was significant at the 5% level. Malonate (0.025 *M*) was without effect on endogenous respiration. Cleland (1950) found only slight inhibition of the succinoxidase of unfertilized oyster eggs by malonate.

Sodium diethyldithiocarbamate. Endogenous respiration was inhibited by diethyldithiocarbamate, the maximum inhibition occurring at 10^{-3} *M* concentration with less inhibition at lower and higher concentrations (Table V). Ethyl carbamate (urethane), an inhibitor of dehydrogenase activity (Keilin, 1925; Stotz and Hastings, 1937), did not affect endogenous respiration in the same concentrations used for diethyldithiocarbamate. Assuming penetration of ethyl carbamate, the difference in action of the ethyl and diethyldithio compounds points to specificity of the sulfur-containing compound and, further, suggests that a copper catalyst may be an important part of the respiratory system.

Discussion

Our objective in this series of experiments has been to establish a background of respiratory data for an understanding of sources of energy and carbonate for shell formation. In so doing we have first described the general respiratory picture of various parts of the mantle of Florida oysters and have found that the pallial zone immediately central to the folded outer margin has a slightly but significantly higher endogenous respiration than either the marginal or central area. It is this pallial zone of the shell which consistently showed the most rapid deposition of calcium in North Carolina oysters previously studied (Wilbur and Jodrey, 1952). As interesting as this apparent correlation may be, the comparison of two sets of data taken at different times and on oysters from different geographical locations must be viewed with caution.

While all steps of the tricarboxylic cycle have not been examined in mantle, enzyme assays and O_2 consumption measurements using several substrates have demonstrated that the major portion of the cycle is present. One apparent difference is the absence of aconitase which catalyzes the conversion of citrate to cis-aconitate and isocitrate. The cycle could operate, however, with isocitrate rather than citrate as the first compound. The isocitrate could then be converted to oxalosuccinate through the action of isocitric dehydrogenase. In homogenates of

the egg of the oyster *Ostrea commercialis* citrate increases oxygen consumption (Cleland, 1951), indicating that in this material citrate is metabolized. While the precursors of the tricarboxylic acid in mantle are not known, pyruvate may well be involved even though its action on respiration could not be clearly demonstrated using strips of mantle. Cleland (1950, 1951) has found that glycolysis and pyruvate occur in the egg of the oyster and the tricarboxylic acid cycle appears to be present for the oxidation of the pyruvate.

The tricarboxylic acid cycle in addition to providing sources of energy would generate CO_2 which might furnish shell carbonate. The respiratory processes described may be expected to apply to other functions of the mantle as well: ciliary movement; mucus formation and secretion; electrolyte regulation; and so on. A direct attack on the relation of respiration to shell formation can be made by measuring the effect of respiratory substrates and inhibitors and combinations of these on shell deposition. (See following paper by Wilbur and Jodrey.)

The very nearly complete blockage of respiration by 2×10^{-4} *M* cyanide points to heavy metal catalysis. However, methylene blue had a negligible effect in reversing the inhibition, suggesting that the cytochrome system may not play a major role in oxidation in this tissue, and, further, that the system concerned may have a redox potential higher than that of methylene blue. A copper catalyst may be involved as indicated by the inhibition of respiration by diethyldithiocarbamate. This can scarcely account for all the oxygen consumption, however, since the maximum inhibition observed was 57%. Maximum inhibition of O_2 consumption by sodium diethyldithiocarbamate occurred at an intermediate concentration of the inhibitor, respiration being less depressed at higher and lower concentrations. Apparently the progressive binding of copper causes the oxygen consumption to decrease to a minimum with a second opposing reaction occurring either as a result of still further decrease in copper concentration or as another effect of the inhibitor. Selenite also exhibited a dual effect on endogenous respiration. With this compound the inhibition at low concentrations was slight and at higher concentration (10^{-3} *M*) there was a stimulation of endogenous respiration. The decrease could be attributed to succinate inhibition, although malonate was without effect on endogenous respiration. Whether selenite at higher concentrations acts in a manner similar to dinitrophenol which also stimulates oxygen consumption in mantle (Maroney and Wilbur, unpublished results) remains to be studied.

Two pieces of evidence in addition to the methylene blue and the diethyldithiocarbamate findings raise the question as to the quantitative importance of the tricarboxylic acid cycle in mantle respiration. Lowering the pH of the medium below 6.6 completely prevented the increased respiration which normally occurs with added succinate without decreasing respiration below the usual endogenous level. Also, malonate left the endogenous respiration unaltered. Both procedures would be expected to decrease O_2 consumption by inhibiting succinate oxidation.

The stability of the oxidative mechanisms of the oyster mantle is noteworthy. The endogenous respiration remained relatively constant in mantles isolated for several days in sea water without added substrate, and these mantles responded to added succinate in the same manner as freshly dissected mantles. The endogenous respiration was also but little changed by altering the pH of the sea water medium from 8.4 to 6.0. Such constancy of respiratory processes would seem to indicate a highly developed control of the intracellular environment. The experi-

mental variations just mentioned may have their counterparts in conditions encountered by the animals such as periods without feeding and variation in the pH of the mantle environment when bathed by sea water as compared with periods when the valves are closed, although in the normal environmental situation the extremes of pH studied here are probably not reached (Dugal, 1939).

Summary

1. The oyster mantle has been studied with respect to endogenous respiration, activity of various oxidative enzymes and response to intermediates of the tricarboxylic acid cycle, and respiratory inhibitors.

2. The endogenous respiration has been found to be similar for different mantle regions, though small differences were present. Endogenous respiration was not significantly altered by changes in the pH of the sea water medium between 6.0 and 8.4 or by isolation of mantles in sea water without added substrate for periods of a few days.

3. Isocitric, succinic and malic dehydrogenases, fumarase, and cytochrome oxidase were present in the mantle. Aconitase could not be demonstrated. Oxaloacetic decarboxylase was found in very high concentration. Its presence may be of significance with respect to the formation of shell carbonate.

4. Isocitrate, succinate, malate, and oxaloacetate stimulated respiration. Enzyme assays and the effects of added substrates indicated the presence of the major portion of the tricarboxylic acid cycle in mantle tissue, although all steps have not been examined. Citrate and pyruvate were without effect on respiration over the range pH 6.0–8.4.

5. Malonate inhibited succinate oxidation partially but did not alter endogenous respiration. Selenite reduced respiration slightly at 10^{-5} *M* but brought about a mean increase of 49% at 10^{-3} *M*. Cyanide, 2×10^{-4} *M*, inhibited respiration almost completely. The inhibition was completely reversible after 1¼ hours but only partially reversible after longer periods. Methylene blue was ineffective in reversing cyanide inhibition.

6. Sodium diethyldithiocarbamate produced a maximum inhibition of 57% at 10^{-3} *M*. Higher and lower concentrations were less effective. Ethyl carbamate (urethane) was without effect at the concentrations employed with diethyldithiocarbamate. The results suggest a copper respiratory catalyst in mantle.

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STUDIES ON SHELL FORMATION. V. THE INHIBITION OF SHELL FORMATION BY CARBONIC ANHYDRASE INHIBITORS [1, 2]

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The formation of mollusc shell involves the deposition of crystalline material, which is largely calcium carbonate, in an organic matrix. The source of the carbonate has been uncertain, although its origin from metabolic CO_2 has been suggested (Robertson, 1941; Sobotka and Kann, 1941). The enzyme carbonic anhydrase is present in the shell-forming mantle tissue of many mollusks (Freeman and Wilbur, 1948); and if the rate-limiting reaction in carbonate deposition were either $CO_2 + H_2O \rightarrow H_2CO_3$ or $CO_2 + OH^- \rightarrow HCO_3^-$, we might expect this enzyme to accelerate deposition (Meldrum and Roughton, 1933). This could be tested by applying carbonic anhydrase inhibitors which should retard carbonate formation. Also, if shell carbonate has its origin in metabolic CO_2, carbonate deposition should be accelerated by supplying the mantle with a substrate that can be readily decarboxylated, provided, of course, that carbonic anhydrase is in excess.

These possibilities have been examined in the oyster, *Crassostrea virginica* (formerly *Ostrea virginica*) by measuring the influence of carbonic anhydrase inhibitors and metabolic substrates on the deposition of radioactive calcium in the shell.

Methods

Oysters were collected in the area of Beaufort, N. C., and maintained in natural waters near the laboratory for several days prior to use at temperatures between 22.8° and 30.0°. The salinity range was 27.6–35.1 except for the period of one week of very low salinity due to a hurricane and during which the oysters were not used. All experiments were carried out within the range 21.5°–25.6°. Individual oysters were prepared as previously described (Wilbur and Jodrey, 1952) and placed in large flat dishes of aerated sea water containing carbonic anhydrase inhibitors for periods of 40 minutes or 7–8 hours. Ca^{45} (4 μc./l.) of high specific activity was then added to the sea water, and the activity of Ca^{45} deposited in the posterior and central regions of the inner shell surface was measured directly (Wilbur and Jodrey, 1952). Isolated mantle preparations with the attached shell (Hirata, 1953; Jodrey, 1953) were employed in studying the effect of respiratory substrates on Ca^{45} deposition both in the presence and absence of carbonic anhydrase inhibitors. Experimental details are presented in the legends of tables.

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Carbonic anhydrase activity was measured in mantle homogenates by the method of Roughton and Booth (1946) as modified by Wilbur and Anderson (1948). Carbonic anhydrase inhibitors were supplied through the kindness of Dr. Emmanuel Waletzky, American Cyanamid Co. The pH of all solutions was adjusted to that of sea water (*ca.* pH 8).

Respiratory measurements on mantle tissue were carried out in oxygen in 5-ml. Warburg flasks using strips of whole mantle weighing 150–220 mg. The thickness of this tissue is such that gas diffusion is not limiting.

Results

Effect of carbonic anhydrase inhibitors on calcium deposition

The effects of 2-benzothiazolesulfonamide (Cpd. I) and 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide (Diamox) (Cpd. II) on the deposition of Ca^{45} are given in Table I. Treatment of oysters with Cpd. I at a concentration of 1 : 20,000 and 1 : 50,000 for 40 minutes prior to the addition of Ca^{45} gave essentially complete inhibition of calcium deposition as shown by the ratio of the deposition with the inhib-

TABLE I

Effect of carbonic anhydrase inhibitors on Ca^{45} deposition in the oyster

	Ratio $\frac{\text{Ca depos.-treated}}{\text{Ca depos.-untreated}}$	Counts/min.		P
		Untreated	Treated	
2-benzothiazole-sulfonamide (Cpd. I)				
1:20,000*	0.02	912±193(12)	41±23(12)	<0.001
1:50,000*	0.07			
1:40,000	0.02	1129±345(8)	43±14(8)	<0.01
1:80,000	0.09			
1:150,000	0.26	1137±261(12)	407±119(11)	<0.05
1:160,000	0.68			
1:250,000	1.11	1189±197(4)	1315±258(4)	<0.8
2-acetylamino-1,3,4-thiadiazole-5-sulfonamide				
1:12,000*	0.60	1243±242(8)	727±178(8)	<0.2
1:25,000*	0.56			
1:100,000	0.52	1550±269(7)	923±219(8)	<0.1
1:150,000	0.71			

Each oyster, connected to a kymograph, was placed in one liter of aerated sea water containing inhibitor. After 40 minutes (starred) or 7–8 hours treatment with the inhibitor 4 μc. Ca^{45} were added. The oyster remained in the radioactive solution for 5 hours. The radioactivity of circular areas of 6.2 $cm.^2$ in diameter on the inner surface of the right valve was measured directly. The radioactivities of a posterior and a central area were averaged to give the value for each individual. The means and standard deviations are shown in the columns under the heading counts/min. The numbers in parenthesis in these same columns indicate the number of individuals. Not all the concentrations of Cpd. II for which data were obtained are given in the table. See text concerning statistical procedures.

itor and in its absence (column 2). With a pretreatment of 7–8 hours, a concentration of 1 : 80,000 caused nearly complete inhibition. At 1 : 160,000 inhibition was still marked but it was not present at 1:250,000. Cpd. II also inhibited calcium deposition. Quantitatively, however, the results are quite different with this compound in that the ratio of deposition with the inhibitor and in its absence fell within the range 0.52–0.71 for all concentrations between 1:12,000 and 1:150,000. As the concentration was increased above 1 : 150,000 the maximum effect occurred at 1 : 100,000, and higher concentrations appeared to give no greater inhibition.

Individual differences in the rate of deposition are always very considerable in oysters, and this is shown in Table I, columns 3 and 4. To demonstrate significant differences between treated and control oysters on a statistical basis the concentration groups have been paired. The P values are given in the last column. With Cpd. II the differences between treated and control groups are not highly significant for single concentrations nor for paired groups. However, when all treated and all control groups are combined the difference is significant at the 1% level.

The marked inhibition of carbonic anhydrase by Cpd. I and Cpd. II (Miller, Dessert and Roblin, 1950) has been demonstrated on oyster mantle using supernates of lightly centrifuged homogenates equivalent to one part of tissue (wet weight) in 50 parts of solution. The enzyme activity at 0° was inhibited completely at a concentration of approximately $1 : 10^7$ for Cpd. I and $1 : 10^6$ for Cpd. II.

Absence of general toxic action of carbonic anhydrase inhibitors

The inhibition of calcium deposition by Cpd. I and Cpd. II might be expected, quite apart from specific enzyme inhibition, if these compounds had a general toxic action. Accordingly, toxicity of the two carbonic anhydrase inhibitors has been studied at various concentrations by recording shell movements and by measuring the oxygen consumption of the mantle. Kymograph records of shell movements were made for all oysters in each experiment. Movements were normal in Cpd. I at 1 : 80,000 which gave almost complete inhibition of calcium deposition. At 1:50,000 abnormal behavior was shown by more frequent opening and closing of the valves followed by increased periods of closure. This behavior became more marked at higher concentrations. Shell movements remained normal in Cpd. II even at a concentration of 1:12,000. The respiration of mantle was unaffected by either compound at the concentrations shown in Table I. A slight inhibition of oxygen consumption (*ca.* 10%) was produced by both compounds at 1:10,000, however. The results on shell movements and oxygen consumption clearly show that concentrations of the carbonic anhydrase inhibitors which had a marked inhibitory action on calcium deposition exerted no general toxic action on the oyster.

Respiratory substrates on calcium deposition

In the Introduction it was suggested that if shell carbonate is derived from metabolic CO_2, then by supplying substrates that can be decarboxylated, deposition of calcium carbonate should be accelerated. The isolated mantle-shell preparation provides an opportunity to put this to test since substrates can be added directly to the mantle and the deposition of Ca^{45} on the shell measured. Succinate, malate,

Because the deposition of calcium by the isolated mantle in the absence of substrate is low, it does not follow, of course, that the same low rate obtains in the whole organism. Nonetheless, in view of the marked effect of oxaloacetate on the isolated mantle it seems likely that in the intact organism CO_2 or substrates that can be decarboxylated, or both, are furnished to the mantle and so increase the rate of calcium deposition. Thus, the mantle, while autonomous in one sense, would only perform effectively because the raw materials of shell are furnished by other parts of the organism.

Summary

1. The carbonic anhydrase inhibitors 2-benzothiazolesulfonamide (Cpd. I) and 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide (Diamox) (Cpd. II) markedly reduced the rate of deposition of calcium in the shell of the oyster *Crassostrea virginica*. Treatment of oysters with Cpd. I, 1:80,000, for 7–8 hours reduced the rate to one-tenth the normal value and gave essentially complete inhibition at higher concentrations. The latter result indicates that inhibition by this compound is not limited to carbonic anhydrase. With Cpd. II the maximum inhibition was 50% and was produced at a concentration of 1:100,000 or higher.

2. Measurements of shell movements and oxygen consumption of mantle tissue showed that concentrations of carbonic anhydrase inhibitors which had a marked inhibitory action on calcium deposition exerted no general toxic action on the oyster.

3. The rate of calcium deposition of the oyster was more than 9 times that of the isolated mantle-shell preparation, confirming an earlier study. The addition of 0.01 *M* oxaloacetate to the mantle-shell preparation increased the deposition rate more than 4-fold, suggesting the utilization of metabolic CO_2 for shell carbonate by the organism. Succinate and malate were without significant effect on calcium deposition.

4. The experimental findings support the view that carbonic anhydrase increases calcium deposition in the oyster when the rate is sufficiently high that either of the following reactions becomes limiting: $CO_2 + H_2O \rightarrow H_2CO_3$; $CO_2 + OH^- \rightarrow HCO_3^-$. At lower rates the enzyme would not be required.

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MITOTIC RESPIRATORY RHYTHMS IN SINGLE EGGS OF PSAMMECHINUS MILIARIS AND OF CIONA INTESTINALIS

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Using the reference (Cartesian) diver technique of Scholander, Claff and Sveinsson (1952), Scholander, Claff, Sveinsson and Scholander (1952) measured respiration of single eggs of marine animals, most of which had previously been studied on somewhat larger samples by Zeuthen (1949, 1950b, 1950c; Cartesian diver method, 1950a). All authors mentioned agree that the rhythm in mitotic division of the fertilized egg is accompanied by a faint respiratory rhythm. However, in the details there are certain discrepancies, especially concerning the reproducibility of the observed rhythm. The discrepancies shall be discussed in this paper which presents new data for single eggs of *Psammechinus miliaris* and of *Ciona intestinalis*. This paper reports all results obtained.

Method

1. Diver

The diver is the same as previously used by the author (1950a), only modified so as to work with one egg instead of a hundred, or a few hundred eggs. The increased sensitivity is obtained 1) by reducing as much as possible the dimensions of the diver and 2) by increasing the sensitivity of the manometer. The diver consists of a chamber and a stopper, both made from the same glass capillary of which two pieces are selected so that one fits tightly into the other. The diver's interior communicates with the exterior through capillary spaces left between stopper and chamber. These spaces permit free equilibration of pressure, and the stopper reduces diffusion. The procedure followed for making and filling this type of diver is illustrated in Figure 1 and is briefly described in the text for that figure. The diver has been drawn too short and thick. For correct dimensions, see inserts of Figures 4 and 6. In Figure 1 also other dimensions are misrepresented.

Before the diver can be filled for an actual experiment its weight must be adjusted so that it will float when a conveniently sized air bubble is introduced into the chamber. For this adjustment a suitable air bubble is pipetted into the newly made diver's chamber, using a Holter braking pipette (step 1 in Fig. 1). The stopper is loosely inserted and the diver is set afloat. It is, however, likely to be far from buoyant. From the rate of sinking or rising in water one soon learns to judge whether or not it shall be possible to regulate it to buoyancy. If prospects are good one first compresses or expands the air bubble by applying pres-

sure or suction to the system. If the diver is brought to float this way the necessary pressure change informs about how much smaller or bigger the air bubble should be chosen next time. Most often, however, it is necessary to remove or to add air and (or) glass. Air is removed or added from the bubble in the chamber, or the stopper's air volume is altered by letting the outer end of the stopper collapse or expand some. This can be done in a surprisingly regulated way by heating in a micro-flame mounted on the stage of the dissecting microscope. If the stopper's outer end is first drawn into a solid tail, the weight of the stopper can be altered by the addition to, or removal of, glass from this tail.

The air volume which finally makes the diver float is measured as the length of an air column in a calibrated Holter braking pipette which is used for all subsequent fillings of this individual diver. The air volume of the floating diver can also be derived from direct measurements of the linear dimensions of the air column in the floating diver.

It does require some skill to prepare these tiny and very fragile divers; many must be discarded. General rules: Always hold the diver with soft and flexible instruments. A piece of rubber, *e.g.*, a rubber stopper with a slit cut in the edge or across the one end, serves as excellent forceps. Watchmaker's forceps protected in the tip with flat pieces of rubber are useful. It is better to hold the divers with the fingers than with unprotected instruments. Capillarity should be made use of whenever possible. In air tiny pieces of glass adhere to wet glass rods and can thus be moved about without much risk of breakage.

2. Manometer

The diver's equilibrium pressure was adjusted and measured using a special "sensitive manometer" (Zeuthen, 1953b). The "manometer" is really a burette (Fig. 2 e, f, g, h), by means of which accurately measured volumes of fluid can be withdrawn from, or injected into, a closed air space (Fig. 2b extending from water surface in a through the ground joint c, the three-way tap d to the water surface e) in which regulated pressure changes are thereby created. The amount withdrawn is read by the movements of the bubble g in the burette. The diver floats in a small amount of alkaline medium in a pocket (a) open to the air space. The "manometer" can be adjusted to the nearest 1/25 mm. water pressure ($\backsim 4 \times 10^{-6}$ atm.) which is somewhat better than the sensitivity of the present diver.

At the beginning of the experiment the pressure in b is adjusted by mouth through n, m, l with d turned 90° relative to the position shown in the figure. With m closed, the manometer k is useful for the fine regulation of the initial flotation pressure in b; n is a CO_2-trap, l is an air brake. During actual measurements the three-way tap d is in the position indicated in the figure.

The volume of the "burette" from 0 cm. to 70 cm. is less than 1% of the volume of b, extending to e. Linear movements of g in the (very uniform) "burette" set up practically proportional pressure changes in b. The system can be reset when g has been moved across the scale. This introduces small changes in the value of h (equation below), which can, however, usually be neglected. The gas exchange is calculated from the formula

$$\frac{\mu l\ O_2}{\text{min.}} = \frac{x \cdot v \cdot V_D \cdot (B + h - e) \cdot 273}{(V + v) \cdot 10{,}300 \cdot (273 + t°)} = x \cdot K$$

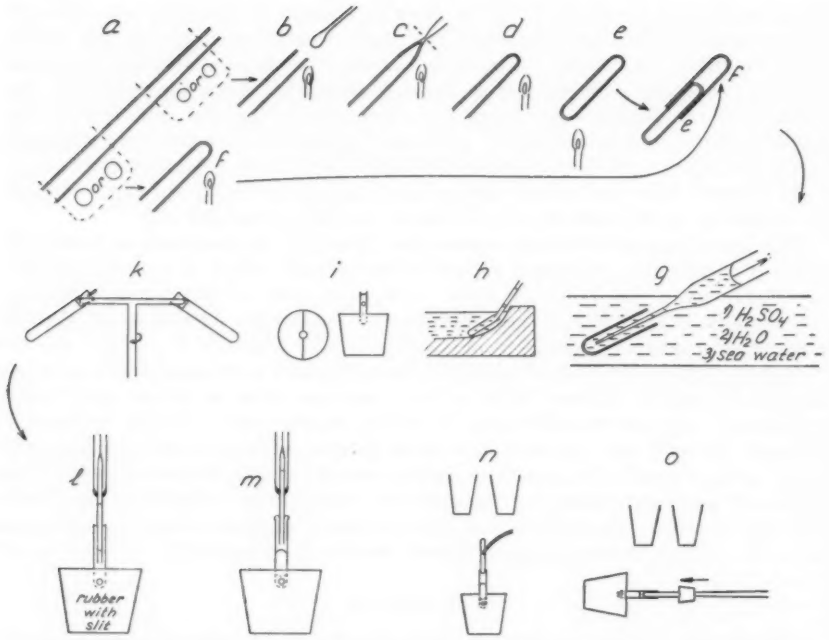

FIGURE 1. Method of making the diver. a. Pull a capillary (Thüringer glass) so as to obtain outside diameter *ca.* 0.2–0.25 mm; inside diameter = outside × 0.87–0.92. If divers made from a given capillary tend to become too heavy or too light, use more thin-walled or more thick-walled glass. Select two pieces of which I fits tightly when pushed a certain distance into II (use head lupe). The cross section of the capillary should be perfectly circular or it should deviate from circular in such a way that I and II can easily be oriented to fit each other. The alternatives are demonstrated. b–e. A micro-flame is mounted on the stage of a dissection microscope. The stopper is sealed—first at what is to be its inner end (upper right, b)—by making contact with a glass rod, pulling, cutting (square line in upper part of c indicates cut) and gently sealing the cut end. This way, a minimum of glass weight is used for sealing. Repeat in what is to be the outer end of the stopper (lower left of e), and seal the far end of the chamber (f). g. The chamber is rinsed in concentrated sulfuric acid and then successively in H_2O and in sea water, always by sucking large amounts of fluid into a pipette, which is introduced deep into the submerged chamber. h–m. The chamber is placed on the slanting side of a dish filled with sea water and the cell is dropped from a Holter braking-pipette into the open end of the diver chamber. The chamber is removed to a holder (i) which is an ordinary laboratory rubber stopper with a slit in it to hold the diver. The rubber stopper is selected to fit the jacket of a small hand centrifuge (k). The cell drops by its own weight to the bottom of the diver chamber or it is spun down with the centrifuge. *Ciona* eggs having a heavy coat of testa cells tend to stick to the glass. They can be pushed down with a small glass rod. The evaporation in the centrifuge is negligible ($< 5\%$). Air is introduced with a braking pipette (l) and, using a second pipette, the water in the upper end of the neck is replaced with alkali, isotonic with the sea water (m). For these operations the pipettes are mounted horizontally and the diver is moved vertically by the use of a special stand (*cf.* Holter, 1943). n. The rubber stopper holding the diver chamber is now placed under the dissection microscope so that the diver is in a vertical position. The stopper (for the diver) is brought to adhere by capillarity to the wet surface of a glass rod. While in the position indicated the glass rod is

in which x is the movement (in mm. per minute) of the bubble g in the "burette," v is the volume (μl) per mm. "burette," V is the volume of b extending to e (c. 100,000 μl; $v/V = 2 \times 10^{-5}$; in $(V + v)$ v can be skipped); V_D is the air volume of the floating diver. B is the barometric pressure in mm. H_2O (760 mm. Hg $\sim$ 10.300 mm. H_2O); h is the initial equilibrium pressure of diver (mm. H_2O) read on k as the difference in height of the menisci (with under-pressure, h negative); e is the vapor tension of water (or of n/10 NaOH and of 2% proteose-peptone) at $t°$ (mm. H_2O).

It should be noticed that x varies inversely with $(B + h - e)$. Therefore the sensitivity of the system should increase greatly at low pressures; and—as long as other factors are not limiting—it should approach infinity when h approaches negative values equal to $B - e$. The present experiments were carried out at total pressures $(B + h)$ varying from 323 to 578 mm. Hg.[1]

There are several possible ways of calibrating the manometer. The one here used is not claimed necessarily to be the best. The space b (from a to d, *cf.* Fig. 2) is determined by weighing with water. The pressure change induced in the whole system (b extending to e, m and meniscus in k), when g is moved from 0 to 70, is read on k, with the meniscus in the left branch adjusted to a defined level. This pressure change should be recalculated to what would have been observed if the space d, to m and k had not been included. The relative volumes of the systems: 1) b to e, m and k, 2) d to e, m and k, and 3) d to m and k (g in all cases in a stable position) are calculated from the pressure changes (read on k) induced for a defined linear movement of the left meniscus in k, when d is in the three positions defining the three spaces mentioned. The data obtained permit the calculation of the pressure change (mm. H_2O) induced in the space b, extending to e, for every mm. movement of g on the scale. In the above formula the calculated pressure change replaces $\frac{v}{V + v}$.

In one bath several "manometers" can be operated simultaneously more or less like Warburg manometers. In the present apparatus there are four manometers but for the present study only one was run at a time.

3. Bath, optical equipment

The bath was a 100-liter tank, on all sides, including the top, well insulated with cork plates. Holes were left open for stirring propeller, for manometer, for illumination from behind, and for observation. The bath was allowed to adjust itself to the room temperature and it varied in the range indicated in Table I, with maximum drifts in the course of an experiment of 0.1°–0.2°/hour.

The flotation vessel remained inserted into the bath for at least one hour before

[1] In this paper only rate curves are presented. It is only on very large-scale cumulative graphs that any scatter of the points can be demonstrated, and that the faint respiratory rhythm can be visually demonstrated. Lack of space prevents the publication of such curves.

turned in a plane perpendicular to the paper so that the cross section of the stopper fits that of the chamber. Then the stopper is fitted into the chamber. Under the dissection microscope with the diver in a horizontal position, it can be pushed further in (o), using the rubber-protected tip of a watchmaker's forceps. The diver, thus filled, is dropped into a dish with alkaline flotation medium and transferred with a pipette to the flotation vessel (Fig. 2a).

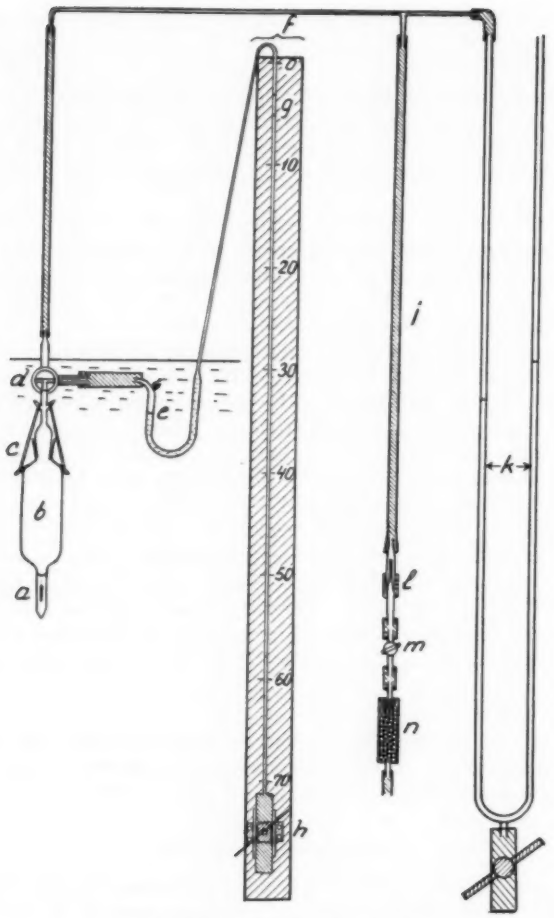

FIGURE 2. Manometer; *cf.* text.

the diver was introduced. This was done as gently as possible and a minimum of water was carried along. This way currents in the flotation medium were minimized during actual measurements, but they were not completely suppressed. It remains uncertain to which extent the initial adjustments in equilibrium pressure observed in most experiments (blanks and runs with egg) should be referred to currents in the flotation medium, to gradual establishment of diffusion steady-states inside the divers, and between flotation medium and gases in the space b, Figure 2, or to as yet unknown shortcomings of the present instrument.[2]

[2] This instrument, as also the 1950 diver, was designed for the demonstration of faint respiratory rhythms. For relative measurements it appears to be unsurpassed, but for absolute measurements it needs to be controlled for background drifts, and to be tested with objects which respire at known rates. Successful work on these lines was carried out by Mr. W. L. M. Geilenkirchen working in this laboratory. The paper will appear in a Dutch journal.

Horizontal microscopes (12.5 × 2.5) with ocular scales are firmly mounted in front of the bath, one for each manometer. The diver's equilibrium pressure is read by "turning point" determinations. The diver is kept floating at about the same level (± 0.1 mm.) throughout the experiment. Whenever a reading is due the manometer is set so that the diver rises slowly. At the same time the respiration makes the diver heavier, and it therefore turns on some level. The manometer, when first set, is not again touched before the diver has turned and the pressure has been read. Figure 3 demonstrates some trials with a respiration diver (1.35 μl), which is subjected to varying initial underpressures. Curves I

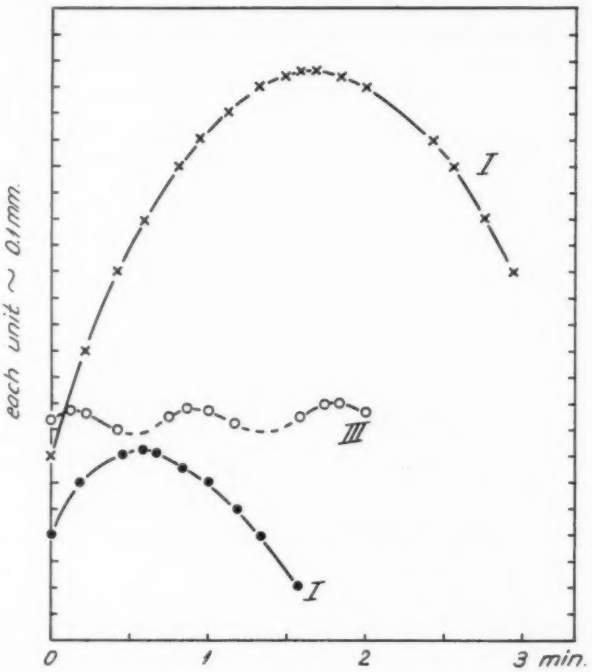

FIGURE 3. Rates of movement of a respiration diver which was subjected to varying initial under-pressures.

represent two cases in which the diver was given relatively low initial pressure and high initial rate of movement. The changing positions are plotted as a function of time. Curve III shows that the level on which the diver turns can be reproduced to about 0.03 mm. and also that the diver can be kept continuously moving up and down within a narrow zone. The results would have been even better if the observer had not had to make notes. Incidentally, one is not forced to accept a reading if the diver happens not to turn on the right level. For the present investigation the measuring periods were about 300 seconds. It is claimed that the turning point can be determined within, say, ± 3 seconds or about ± 1% of the time be-

tween successive readings or better. Close inspection of the data underlying the curves of Figure 3 indicates that about 0.005 mm. displacement of the diver was enough for the observer to decide about the direction of the diver's movement. In the optical field this corresponds to 0.15-mm. displacements. Suitable reference marks on the diver are points which in the beam of light appear like little stars. If such a point happens to form a continuation of one of the lines of the ocular scale the decision is about whether the line extended with the "star" tends to bend up or down in the optical field.

4. *Material*

The eggs of *Ciona intestinalis,* fertilized and unfertilized, were used. The testa cells were not removed; they have been found to make up for 20–30% of the res-

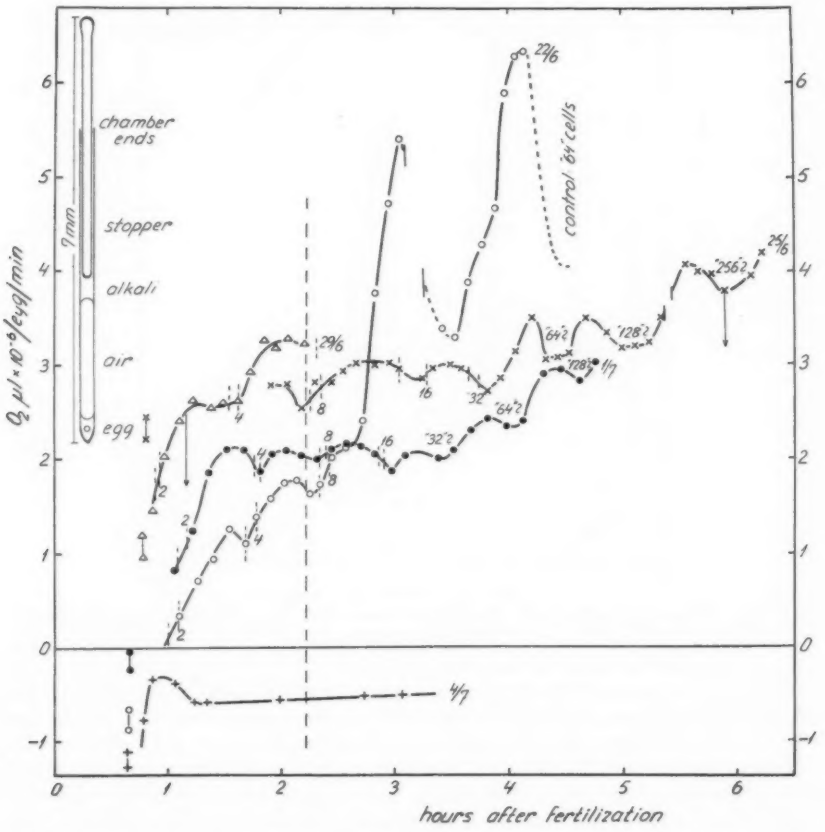

FIGURE 4. Four experiments on single eggs of *Psammechinus miliaris,* with one control experiment (4/7); *cf.* Table I and the text.

piration of the unfertilized egg (Holter and Zeuthen, 1944) and less in the case of the fertilized egg. The eggs were thoroughly washed before use. Another object used was the fertilized egg of *Psammechinus miliaris*, Z-form. The jelly was removed with a pipette, the fertilization membrane was retained. Both animals are from the littoral zone and the experiments were run in sea water, salinity 20–25‰. The divers were floating in 22‰ NaCl, made up to n/5 with isotonic NaOH; no Na-taurocholate was added.

Experiments

A. *Psammechinus miliaris*. Figure 4 shows four experiments with single eggs of *Psammechinus*. The same diver was used throughout, and an experiment with empty diver is also reported (4/7). The ordinate shows the rate of oxygen uptake per egg per minute, and the abscissa indicates hours after fertilization.

Table I

Oxygen consumption in single eggs of Psammechinus miliaris. Data pertaining to Figures 4–6

		$B+h$ (mm. Hg)	$t_1°$	$t_2°$	O_2/min., µl (135 min.)	Vol./egg, µl	µl O_2/µl egg/min.
	22/6	340	16.9	17.3	2.2×10^{-6}	6.25×10^{-4}	3.5×10^{-3}
	25/6	450	18.2	17.7	3.2×10^{-6}	4.9×10^{-4}	6.5×10^{-3}
I	29/6	533	19.6	19.8	3.8×10^{-6}	5.25×10^{-4}	7.2×10^{-3}
	1/7	418	18.6	19.3	2.6×10^{-6}	4.8×10^{-4}	5.4×10^{-3}
	4/7	344	21.1	—	—	—	—
	20/6	538	16.9	17.4	2.6×10^{-6}	5.0×10^{-4}	5.2×10^{-3}
II	24/6	523	17.5	17.5	2.1×10^{-6}	4.75×10^{-4}	4.4×10^{-3}
	7/7	578	19.9	20.0	3.2×10^{-6}	—	—
III	4/7	362	—	—	—	—	—
Aver.		466	18.5		2.8×10^{-6}	5.2×10^{-4}	5.4×10^{-3}

I, fertilized eggs, Figure 4. II, fertilized eggs, Figure 5. III, control experiment, Figures 4 and 5.

Two points connected with a vertical line indicate the time when the diver was initially brought to float in the flotation medium. The position of these pairs of points on the ordinate is in all cases arbitrary. Due to space limitation two curves (three in Fig. 6) have been displaced on the ordinate to the extent indicated by the length of the arrows. Gaps on these curves (and on other curves presented) indicate resetting of the manometer. Important characteristics of the 5 experiments are given in Table I. The two temperatures are at (or near) the beginning and the end of each experiment.

The diver, shown with typical filling in the insert of Figure 4, has a gas volume (V_D) of 0.063–0.065 µl, determined slightly differently, in the 5 experiments. The internal cross sectional area (A) of the diver capillary was calculated from several diameters and V_D was calculated from the formula $A\cdot(l-2r)+4/3\pi r^3$ in which l is the length of the air column from meniscus to meniscus and $2r$ is the (average) diameter of the diver capillary; l was measured on the floating diver. In the graphs

pairs of vertical stippled lines across a curve indicate the time when a division was beginning and when it was completed. The number of cells resulting from a division is indicated. After the 16-cell stage (32-cell stage on 25/6) accurate observations were no longer possible. The numbers placed by the time of later minima on the curves represent guesses. We actually know (*cf.* Zeuthen, 1951, 1953a) that "64," "128" and "256" cell stages do not exist. The cell numbers are in all cases lower than indicated. The long stippled line (2 h. 15 min.) shows the time when the absolute rates reported in Table I were measured as the distance from curve to curve (4/7) for the control experiment. Egg volumes were calculated from the measured diameter of the experimental egg itself, or from the average diameter of several control eggs.

The position of the control curve (4/7) indicates that probably in all cases there is some diffusion of gas into the diver from the surroundings. Unfortunately, the diver was broken before another control was run, and this causes some uncertainty as to the absolute rates measured. However, Borei (1948) for both *Psammechinus* forms ("Z" and "S") found an average rate of O_2 uptake of 3.1×10^{-6} μl/egg/min. (18°, 120 min. after fert., egg size 4.43×10^{-4} μl). The results here reported check well with this. Inspection of the rate curve 22/6 for slope indicates that we have reason to suspect the value (Table I) for this experiment to be too low.

The egg used on 25/6 stayed in the diver until the mesenchyme-blastula stage; the eggs used on 22/6 and on 1/7 were removed immediately after the experiment and not observed further. The egg studied on 29/6 stopped development by the time of the third division. By this time the experiment had to be discontinued because the stirring motor set up disturbing vibrations; two hours later the four blastomeres of this egg had fused into two. By this time most of the control eggs were blastulae, but a number had developed into monsters of the same type as the one in the diver.

Figure 5 presents two more experiments with single *Psammechinus* eggs, both showing asynchrony between divisions of the two first blastomeres. The egg used on 20/6 showed a mild degree of asynchrony, the second division being delayed 3–4 minutes in the one blastomere. In the following division cycle this delay could again be observed; 7 cells were directly counted—there may perhaps have been 8. In this experiment optical conditions were poor. Possible division activity was noted at times indicated (?). On the following morning this egg was a mesenchyme blastula (while still in the diver); the controls were gastrulating. The blastomeres in the egg studied on 24/6 showed pronounced asynchrony of divisions. The number of cells observed at any time is indicated, optical conditions were good. Both experiments (20/6, and especially 24/6) are inconclusive as to respiratory cycling, but they may indicate that pathological conditions which bring about asynchrony of division may at the same time increase the amplitude of the respiratory cycles above normal.

Such a situation, if correct, suggests that physiological interaction between neighboring cells which are in different phases of mitosis might result in increased cycling amplitude. This hypothesis was put to a preliminary test, with negative result, in the experiment of 7/7. For this experiment a good batch of eggs was divided into two lots and fertilized with a time interval of 17 minutes, which roughly equals half of one mitotic cycle. The jelly, and in most eggs also the

fertilization membrane, was removed shortly after the fertilization using a narrow pipette. From the lot first fertilized, 92 eggs in the 2-cell stage were selected, 44 without membrane and 48 with. From the second lot 92 eggs were isolated in the 2-cell stage, all without membrane. All 184 eggs were thoroughly mixed together and introduced into a diver (gas space: 9.8 μl). The resulting respiration curve (7/7) is indicative of simple interference between two groups of eggs, each of which shows respiratory cycling of about the magnitude shown in the typical curves of Figure 4. Thus, this experiment supplied no evidence of physiological

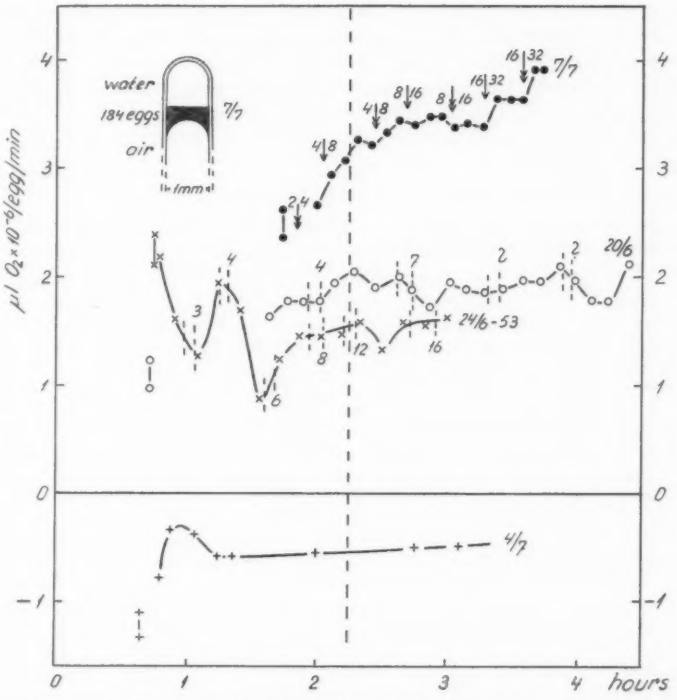

FIGURE 5. Two deviating experiments (20/6, 24/6) on single *Psammechinus miliaris* eggs. One experiment (7/7) with 184 eggs fertilized in two separate lots (92 + 92 eggs) showing absence of physiological interaction between the two groups.

interactions between two groups of cells which are much out of phase. In Figure 5 single and double arrows indicate the approximate division times of control eggs belonging to the two separate lots.

The absolute respiration rates measured two hours 15 minutes after fertilization are given in Table I. The experiment 4/7 serves as base line for the experiments on 20/6 and 24/6. The base line for the experiment 7/7 is the 0-line.

B. *Ciona intestinalis.* The diver used for these experiments looked much the same as the one used in *A;* however, all dimensions were slightly larger. V_D

measured as under *A* averaged 0.112 μl (variation 0.110–0.115, for Experiment 8/6 a mistake was made when the dimensions were measured—the calibration found on earlier days was accepted). In Figure 6 rate of oxygen uptake is plotted on an arbitrary time scale. Time for beginning and for end of each division is indicated with stippled vertical lines, and the resulting number of cells is indicated; (?) indicates poor conditions of observation. The experiments on 11/6 and 5/6 are for unfertilized eggs, and the experiments on 3/6, 6/6 (2 exps.) and 8/6 are on fertilized eggs which all showed beautiful development in the diver (not observed beyond the time of experiment).

The two control experiments on 10/6 were made with the same diver as used for all *Ciona* experiments, but with certain changes introduced by accident. After completion of the experiment on 8/6 some of the outer end of the diver chamber broke off. Therefore the diver became buoyant with a reduced value of V_D (0.060 μl instead of 0.112) and the diffusion barrier set up in the diver's neck after introduction of the stopper became shorter, *i.e.*, probably less efficient. Before the accidental loss of glass the stopper fitted the diver chamber over a distance of 3.75 mm. This distance was reduced to about 3.1 mm. The diver thus modified was floating at a pressure not too different from that used in the biological experiments (*cf.* Table II) and the readings represented by the hollow circles were obtained. Then the diver was removed from the vessel and the stopper was pulled about one mm. back in order still further to reduce the diffusion barrier in the diver's neck. The diver was set afloat again and the readings represented by black circles were obtained. The (small) difference in level between the control curves and the 0-line is accepted as representing a maximum of diffusion of gas into this diver in all experiments.

TABLE II

Oxygen consumption in single eggs of Ciona intestinalis. Data pertaining to Figure 6

		$B+h$ (mm. Hg)	$t_1°$	$t_2°$	O_2/min., μl (1 hour)	Vol./egg, μl	μl O_2/μl egg/min.
I	3/6	473	18.32	18.50	1.05×10^{-5}	2.4×10^{-3}	4.35×10^{-3}
	6/6(1)	455	18.52	18.55	1.03×10^{-5}	—	—
	6/6(2)	506	18.55	18.75	1.22×10^{-5}	2.35×10^{-3}	4.7×10^{-3}
	8/6	463	17.88	18.22	1.14×10^{-5}	2.3×10^{-3}	4.65×10^{-3}
II	5/6	570	18.21	18.35	0.83×10^{-5}	—	—
	11/6	323	—	19.83	0.75×10^{-5}	2.0×10^{-3}	3.75×10^{-3}
III	10/6	460	—	—	—	—	—

I, Fertilized eggs. II, Unfertilized eggs. III, Control exp.

In Table II the respiration intensity for each egg is measured (at time one hour) by the distance between experimental and control curves. Also the cell volume is given, based on direct measurements of two cell diameters or on an average diameter for at least four control eggs.

The respiration intensity here measured for single eggs in early cleavage stages

(1.0–1.2 × 10^{-5} μl/egg/min.) compares well with results reported in 1944 for single *Ciona* eggs by Holter and Zeuthen (*ca.* 1 × 10^{-5} μl/egg/min. at 22.5°), also when temperature differences and possible volume differences between eggs are taken into account. The present experiments were run at a 4° lower temperature than used in 1944 but with eggs which on an average seem to have been slightly bigger than in 1944. Also for the unfertilized eggs the over-all rates check with the earlier report.

Discussion

Out of a total of 10 experiments on single dividing marine eggs (4 *Ciona* eggs, 6 *Psammechinus* eggs) 6 showed respiratory cycling of much the same kind (*Psammechinus* 25/6, 29/6, 1/7, Ciona 3/6, 6/6 (2), 8/6). A seventh (*Psammechinus* 22/6) showed the same kind of cycling during divisions 2 and 3. The atypical experiments are the following: *Psammechinus:* one experiment (24/6) showed strong respiratory variation before the 8-cell stage, and one (22/6) considerable variations between 16- and 64-cell stages; one experiment (20/6) was inconclusive. One *Ciona* experiment (6/6 (1)) showed considerable early variations, but was interrupted early.

Concerning the deviating experiments it should be stressed that sometimes (*cf.* control 4/7) readings within the first hour or so are uncertain. The reasons are imperfectly understood. However, the always possible presence of such technical difficulties places considerable doubt on all non-reproducible deviations from the general findings, and the author is therefore unable to decide whether the results on 6/6 (1) and on 24/6 should be referred to errors in the technique or to the respiring cells themselves. On 22/6 the curve for the early part of the experiment seems to indicate stability of the system. However, by about the time of the 4th division the respiratory rate suddenly increased steeply and the respiration ended up so intense that the scale was used up after four more readings. Then the manometer was reset, *i.e.*, the pressure in the system was lowered and the diver was equilibrated with the indicator air bubble back in the upper end of the scale. The two first readings after this showed low rate of respiration but then the rate accelerated much the same way as before. Again, one is unable to decide between the egg or deficiencies in the apparatus as the cause. A leakage developing in stop-cock d, Figure 2, and re-developing after the stop-cock was operated with the resetting of the manometer, *might* perhaps result in effects as those recorded.

For *Ciona* two experiments on unfertilized eggs are available. One of the two (11/6) might perhaps be fitted with a cycling curve, but the author feels that the least committing interpretation is absence of cycling. There are independent reasons for not depending too much on the experiment on 11/6. It was run at such a low pressure (*cf.* Table II) that the manometer had to be reset too often, as demonstrated by the many gaps on the curve.

Had all runs on *Ciona* eggs, fertilized and unfertilized, been fitted with smooth, non-cycling curves, we should evidently have found more scattering around the curves for dividing, fertilized eggs than for non-dividing, unfertilized eggs. This suggests that the respiration in fertilized eggs is subject to variations not present in unfertilized eggs, and where possible the curves are drawn to suggest that these variations are rhythmic and timed with the mitoses.

It seems doubtful whether the present techniques—as also the one of Scholander *et al.* (1952)—is reliable enough accurately to record the respiratory variations in every run, and many more control experiments with unfertilized eggs would have been necessary if we were to predict the frequency with which an experiment with fertilized egg would fail to demonstrate a rhythm actually present. It is strongly emphasized that in this situation the degree of reproducibility of the rhythmic phenomenon itself remains our main control. The type of cycling respiration which is demonstrated throughout the curves for the experiments on 25/6, 29/6, 1/7, in part of the curve 22/6 (all for *Psammechinus*) and on 3/6, 6/6 (2) and 8/6 (for *Ciona*) the author considers as reproducible as can be expected in view of the technical difficulties involved. They largely confirm results he has previously obtained on single frog eggs and in mass runs on different marine eggs. All deviating observations, discussed in detail above, he considers interesting but quite inconclusive.

Scholander *et al.* (1952) published a material of 19 runs on four different species of single dividing eggs which were studied for rhythmic respiration. As far as can be gathered from the description of Scholander *et al.* a few eggs show definite respiratory cycling, one even very strong cycling; 10–11 eggs show definite cycling with an amplitude much the same as found by the present author. However, the authors state that it almost vanishes after the two or three first divisions. Five experiments are inconclusive as to cycling, or it is concluded that there is no cycling. Of these 5, the 4 were with Urechis eggs which show the lowest rate of respiration of the eggs studied. The most constant cycling was in single Dendraster eggs which on an average respire more intensely than any of the other three species of eggs studied. One exceptional case of an egg (*Strongylocentrotus purpuratus*) showing abnormally intense cycling is suggestive. However, this egg was definitely sick. It developed very slowly and in an asynchronous way (*cf.* Fig. 5 of this paper).

On the basis of this material Scholander *et al.* concluded that respiratory cycling is observed, but (p. 197): "We must emphasize, however, that cell division in individual cells very often takes place without any demonstrable cycling. Quite regularly, therefore, the energy requirements for the different phases are apparently fitted nicely together within the limits of a steady constant flow of oxidative processes." In the absence of experiments with objects which can be expected to show non-rhythmic respiration the reproducibility of any type of rhythmicity is the only control, and to the present author the stated absence of such reproducibility in the material of Scholander *et al.* does not invite conclusions as the above quoted. First, proof is required that the method will reproduce constant rhythmicity, if present, in every run. Substantiated doubts whether the method of Scholander *et al.* will do this have been expressed on a previous occasion (Zeuthen, 1953a). To the present author all evidence, viewed together, indicates as good reproducibility as for technical reasons can be expected of the phenomenon of respiratory cycling in cleaving eggs.

Scholander *et al.* also conclude (p. 197) that: "In our experiments the cycling is always strongly damped. It almost vanishes after the two or three first divisions." They further write (p. 197): "Zeuthen (1949) has found that later on cycling increases very markedly. Even if it can be demonstrated that this late cycling also occurs in single eggs, there will still be a minimum of cycling after

the second or third division, and hence there can be no simple correlation between the wave amplitude and the steadily increasing number of cell divisions." The views previously expressed by Zeuthen (1949) are these (p. 316): Up to the stage of division 4–5 in *Psammechinus miliaris*, division 6–7 in *Dendraster excentricus* and in *Strongylocentrotus franciscanus*, limit unknown for frog and Urechis the extra respiration is a function of the constant mass of the embryo rather than of the number of cells dividing. But after this stage has been passed the extra respiration becomes more closely a function of the number of cells dividing than of the mass of the embryo. The "extra respiration" of each division was

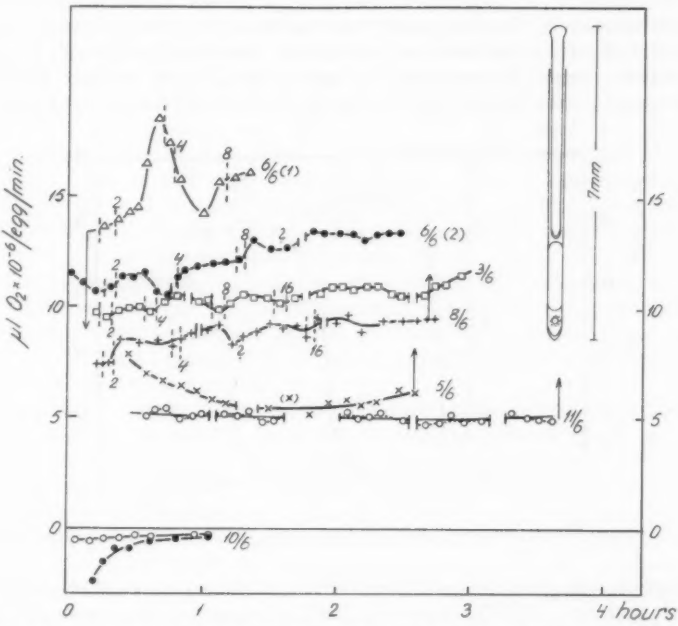

FIGURE 6. Four experiments (3/6, 6/6 (1), 6/6 (2), 8/6) on fertilized dividing *Ciona* eggs, two (5/6, 11/6) on unfertilized eggs, and two control runs (10/6) without eggs.

defined as the small areas, from one minimum on the respiration curve to the next, between the respiration curve and a smooth auxiliary line drawn to touch all minima.

The first half of the above statement, about constancy of the respiratory cycling in the early divisions, is confirmed by the present experiments. The author finds no evidence of decreased cycling after the first 2–3 divisions. On the other hand, the increased cycling with the late divisions is only slight. However, it was commented already in 1949 that in this latter respect different eggs behave differently. In the situation now established it should be pointed out that within the single egg asynchrony of divisions begins with divisions 4–5 and tends to become pro-

gressively worse later, even though a statistical rhythm persists throughout cleavage (*cf.* Zeuthen, 1951; Holter and Zeuthen, 1955). Slightly increased respiratory cycling by the time when asynchrony sets in is therefore indicative of definitely increased cycling in the single blastomeres.

Further discussing a point raised by Scholander *et al.*, Claff (1953) expressed doubts about one of Zeuthen's main summary points (1951) which is (p. 66): "In the segmenting egg the oxygen consumption follows a rhythm which throughout segmentation correlates with the mitoses." However, he quotes evidence only from 1949 and 1950, not from the paper from which the summary point is taken. This evidence (Zeuthen, 1951; Holter and Zeuthen, 1955) is the following: Mitotic rhythmicity occurring throughout 10 successive cycles was demonstrated in batches from single females of *Psammechinus microtuberculatus*. It was stated that increasing rates of metabolism occurred whenever a considerable majority of the cells were in mitotic stages characterized by having nuclei with nuclear membranes. For single eggs it was stated that asynchrony between divisions progresses from

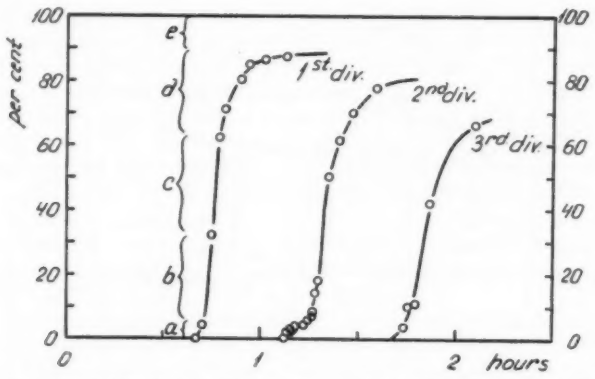

FIGURE 7. Unequal distribution of division frequencies in the 1953 Kristineberg material.

16-cell stage on, but a manifest statistical mitotic rhythm persists throughout cleavage. Scott and Fox (1952), quoted by Claff in support of his views, do not disprove the presence of a statistical division rhythm throughout cleavage in batches of Arbacia eggs, and they did not measure respiration. Actually, their findings can very well be interpreted to agree with Zeuthen's data (1951) demonstrating a statistical division rhythm.

Nevertheless, it is of course true that the degree of synchronization in batches cannot be expected always to be equally good. During the summer 1953 the author had intended to do mass runs on *Psammechinus miliaris*, Kristineberg Z-form, and to study the influence of inhibitors on the respiratory rhythmicity. However, respiratory cycling was only occasionally observed. This was nothing new or surprising because it happens whenever the egg material shows poor synchronization of cleavage. Usually this difficulty can be overcome by selection of suitable females, but not this year.

The degree of synchronization of divisions was then investigated in the follow-

ing way. From a batch of fertilized eggs (one female) showing more than 90% fertilization, with nicely lifted membranes, 100 fertilized eggs were selected with a Holter braking pipette within three minutes after fertilization. The curves of Figure 7 give the relation between time after fertilization and per cent of cells entering the first, the second, and the third division. The three curves demonstrate progressively more and more unequal distribution of division frequencies, with 30% being excessively delayed already in the first division; the number of eggs which at all divide decreases with each new division. The eggs entering the first division were separated into five groups (a–e incl.), a representing those which first enter division, d those which cleave with most delay. Group e (seven cells) was discarded. These eggs divided directly into four cells. Whereas group a developed nicely to swimming blastulae, groups b, c, d stopped development at earlier stages and the earlier, the more first division was delayed. Six hours after fertilization all eggs in group a (four eggs) were young blastulae (swimming next morning); in group b (28 eggs) after six hours fourteen were young blastulae, six less advanced "morulae" and eight were eight-cells. Next morning thirteen were swimming blastulae, the others had not advanced further. Six hours after fertilization, group c (31 eggs) were "morulae" and did not develop further; group d (30 eggs) all remained in division stages with anything from one to eight cells. This type of experiment was repeated several times with comparable results, but the one here reported was the most striking. These observations present very interesting problems but could not be followed up because our time at the zoological station was up. It should be stated that the spawning season was not yet over; also that exceptional climatic conditions this year (extreme heat with surface temperatures approaching 25°) influenced the eggs strongly. Everybody complained about "poor material."

The curves of Figure 7 demonstrate heterogeneities in the 1953 Kristineberg material which unquestionably represent extreme tendencies which may or may not exist in all batches of sea urchin eggs. The 1947 *Psammechinus* material used at Kristineberg by the author in his first experiments on respiratory rhythms in dividing eggs was definitely better than the 1953 eggs. Nevertheless, already in 1947 it was found useful (*cf.* Zeuthen, 1950a) to select small batches of eggs for experiments as those individuals in a population (one female) which first enter first division. This secured for the 1947 respiration experiments relatively synchronously developing small groups of eggs. In later experiments on other sea urchins and on Urechis no such selection was made for the reason that representative small samples of whole batches from "good females" (picked on basis of quality of membrane lifting and of percentage fertilization) showed reproducible respiratory cycling throughout cleavage. The author accepts as likely that the eggs of those species for which the nicest respiratory cycling was observed in mass runs may also have been those which showed the highest degree of synchronization of development in whole batches. On this basis he deems *Dendraster excentricus* (1949) and *Urechis caupo* (1949, 1950c) the best material with which he has worked. Still, it was for the Naples *Psammechinus microtuberculatus* that respiratory cycling and mitotic activity was demonstrated to correlate throughout cleavage (Zeuthen, 1951).

In the following the situation demonstrated for the 1953 Kristineberg material

shall be considered extreme and atypical. We shall accept that usually (*cf.* Blum and Price, 1950 for *Arbacia punctulata*) there is a normal distribution in time of the frequencies with which eggs in a population from one female enter every new division and we shall further, for the sake of simplicity, assume that all eggs in a small population show identical respiratory variations only with some displacement of the curves in time. How much, then, shall the excursions on the respiration curves found for single eggs be blurred in records for the whole population?

In Figure 8, I, the curve for *Psammechinus*, July first, was accepted as common for all eggs in a small population of 5. This curve was re-plotted five times (a) at time intervals of three minutes, which means that there is a difference in time of twelve minutes between the fastest and the slowest egg. A full cycle lasts anywhere from twenty-eight to forty minutes. The five curves are then mixed (b, heavy curve) to give the respiration curve for the population for comparison with the curve for a single egg. In Figure 8, IIb, the same procedure is repeated, only with the curves plotted five minutes apart (twenty minutes between fastest and slowest). In the cases of Figure 8, I, II, the divisions are equally, not normally, distributed in time, *cf.* distribution diagrams; a more natural situation is constructed in Figure 8, III. In this case the two heavy curves from Figure 8, Ib, IIb, are mixed with one curve for a single egg. The resulting heavy curve for this population is indicated. In this population there is reasonably good approximation to a normal distribution of identical stages in time (*cf.* diagram to the right in 8, III). The striking fact is that although in Figure 8, III, the fastest and the slowest cells are separated by a time corresponding to 5/7 of the shortest cycles, all respiratory variations for the single eggs are recorded in the population, but of course with some damping of the wave amplitude, especially in the case of the shortest cycles. In Figure 8, III, the abscissa is chosen so that zero corresponds to zero consumption. The respiratory variations observed by the author for single frog eggs (1946) and later for early divisions in small populations of marine eggs are very much alike when shape and amplitude (*ca.* 5% of total rate) are concerned. Linderstrøm-Lang's calculations (1946) demonstrated that the records for a single frog egg may have been subject to some damping caused by diffusion in this fairly big system. The true variations may have occurred with an amplitude of 7–11%. In the case of the marine eggs the diffusion factor can hardly have been of significance (Zeuthen, 1950c), but as demonstrated in this paper there may have been damping of about the same order of magnitude (slightly different from one group of experiments to another) as in the frog egg experiments, however due to incomplete synchronization. It is now considered that the curves here published for single marine eggs (Figs. 4 and 6) represent very close approximations to actual intracellular variations in the rate of oxidation, typical probably of many types of dividing egg cells.

Figures 4 and 6 show that it is no longer possible to describe the respiration of dividing eggs as following a sine curve. Clearly, in *Psammechinus miliaris* and in *Ciona intestinalis* there is a rather sharp dip in the rate of oxygen consumption reaching a minimum around the time of every new division, especially divisions 2–5 inclusive, and rapidly again increasing after the division. With later divisions the sharp dips change into broad valleys. The respiratory rate tends to remain on a high level for some time prior to the next dip, especially so in the early

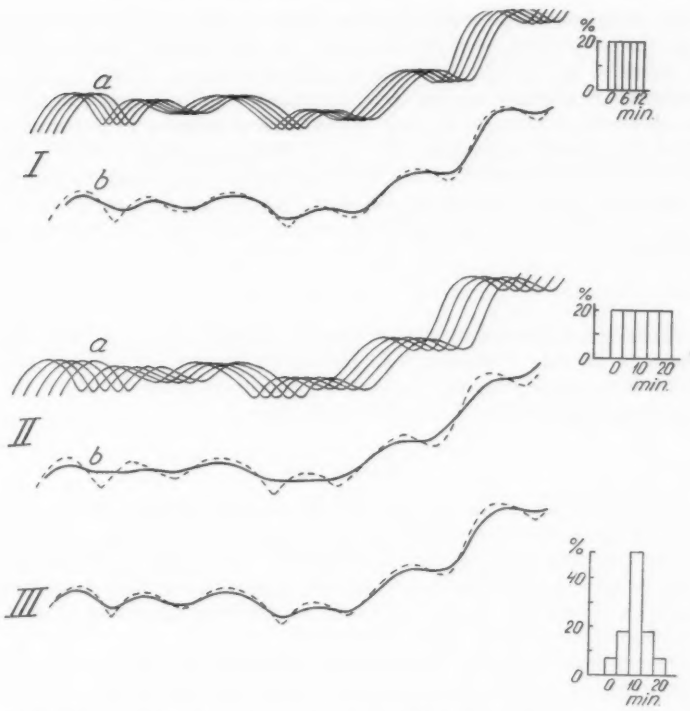

FIGURE 8. Construction of average curves for populations (heavy curves Ib, IIb, III) of eggs which are assumed to respire according to identical curves, only with some displacement in time. The dotted curves in Ib, IIb, and III are identical with the one curve replotted 15 times in Ia, IIa. The diagrams to the left in the figure show the frequencies with which the cells in the populations I, II and III enter every new division.

divisions. The cell division sometimes begins already when the respiration is decreasing, more often when it is minimum or already increasing. This confirms most of the previous results of the author (frog (1946) sea urchins (1946, 1949, 1950b, 1951) the ciliate *Tetrahymena piriformis* (1953b)) and of Scholander *et al.* (1952) (sea urchins, *Urechis*). In sea urchins, the respiratory rate (*cf.* Zeuthen, 1951; Holter and Zeuthen, 1955) seems to be minimum around late telophase, it increases sharply during inter- and prophase, remains level around mid-mitosis and drops again in late mitotic phases. Sometimes, however, the division has been observed to occur early in the phase of decreasing respiratory rate. This was for a few (not all) runs on several hundreds of *Urechis* eggs (1950c); however, in this case conditions for optical observations were admittedly much poorer than in runs with single eggs. If confirmed, this finding warns us that the respiratory cycling may appear to be more closely associated with other mitotic events than with the cytoplasmic fission (*cf,* discussion in Zeuthen, 1951; Holter and Zeuthen, 1955).

This view is corroborated by the following facts: A single fertilized frog egg which altogether failed to divide showed normal respiratory cycling (1946) and colchicin-treated fertilized eggs of *Psammechinus microtuberculatus* (1951) which did not divide showed respiratory cycling with increased amplitude and extended period.

The observed respiratory rhythm shows inverse relation to the state of development of the Mitotic Apparatus (M.A.) of Mazia and Dan (1952). The M.A. is a structure which is tied together by S-S-bridges. This may be highly important, suggesting some connection between SH-groups and respiratory cycling. However, for several reasons it is too early to make any deductions from this observation.

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Summary

1. In single fertilized marine eggs (*Psammechinus miliaris, Ciona intestinalis*) the rate of respiration shows a temporary decrease (5–10%) and reaches a minimum every time the egg divides. The findings were reproduced in 6½ experiments out of 10. The method is not yet well enough controlled to permit definite statements as to whether or not deviating experiments should be referred to insufficient technique or to the eggs themselves.

2. The respiratory rhythm demonstrated resembles the one previously observed in the frog egg, in the *Urechis* egg and in several echinoderm eggs.

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